SEROLOGICAL CHARACTERIZATION OF *TRYPANOSOMA (NANNOMONAS) CONGOLENSE* USING *IN VITRO*-DERIVED METACYCLIC FORMS

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ABSTRACT

This thesis describes the development and application of techniques to characterize the metacyclic variable antigen type (M-VAT) repertoires of stocks of Trypanosoma congolense which were isolated from a defined geographical area. Seventeen stocks of T. congolense were isolated from domestic dogs in a 320 hectare area around Kakumbi, Chipata District, Zambia. Six of these stocks were cloned and stabilated as TREU 1881, TREU 1885, TREU 1894, TREU 1896, TREU 2034 and TREU 2037. Insect form in vitro cultures were initiated from the proboscides, proventriculi or midguts of tsetse flies infected with the cloned stocks. The cultures produced antigenically stable metacyclic trypanosomes in large numbers on a regular basis.

In vitro-derived metacyclic forms were used as reference antigens in four serological assays. Firstly, monoclonal antibodies were prepared to the metacyclic surface antigens of one stock, T. congolense TREU 1885. Nine monoclonal antibodies were produced which, individually, recognized between 6% and 18% of the metacyclic population. When pooled, the monoclonal antibodies stained 70-84% of the entire M-VAT repertoire. The monoclonal antibodies were used in an indirect fluorescence antibody test (IFAT) to determine whether M-VATs which occurred in TREU 1885 were present in the metacyclic populations of the other stocks in culture. None of the M-VATs present in the other in vitro-derived stocks were recognized by the monoclonal antibodies indicating that TREU 1885 M-VATs were distinct and characteristic for that stock.

A cross protection assay was carried out in mice using viable, in vitro-derived trypanosomes. Groups of mice were infected with each of the six stocks, then treated 10-14 days post-infection with either diminazene aceturate or isometamidium chloride. Fourteen days post-treatment, each group was challenged with one of the six stocks. The animals were immune to challenge only against the infecting stock indicating that the six stocks cultured in vitro were antigenically distinct.

In order to facilitate serological typing of the 17 stocks, an enzyme-linked immunosorbent assay (ELISA) and IFAT were developed using glutaraldehyde- and formalin-fixed intact trypanosomes respectively. Twenty-one day post-infection antisera from rabbits were used to identify M-VAT specific immune responses to the metacyclics of the cultured stocks. The ELISA, although shown to be M-VAT specific in early experiments, failed to distinguish between antigenically distinct stocks of the reference collection.

A triple labelling IFAT which detected the IgG-specific immune response of the host was shown to be the most effective technique for distinguishing M-VAT repertoires using fixed trypanosomes. Using this assay, sera raised against the 17 original isolates were examined using in vitro-derived metacyclic trypanosomes from the six cloned stocks as antigens. Fluorescence was observed only between known homologous antisera and trypanosomes thus indicating that from 17 stocks, at least seven serodemes were present in this area.

The reasons for the wide diversity in serodeme distribution in this small area are discussed in relation to the tsetse fly and game animal populations. The value of serodeme characterization and its

use in determining the ecology and natural history of *T. congolense* and relevance to strategies for implementing rational control measures are also discussed.

CHAPTER ONE

GENERAL INTRODUCTION

Trypanosomes are haemoflagellate parasitic protozoa belonging to the genus Trypanosoma Gruby, 1843 and have been found in every class of vertebrate from fish to mammals. The majority of trypanosome species are non-pathogenic and their vertebrate hosts are capable of withstanding infection without developing any apparent symptoms. However, some species are pathogenic to man and his domesticated livestock and have considerable importance in human and veterinary medicine. Trypanosomes pathogenic to man are found in South America (Trypanosoma cruzi) and Africa (T. b. rhodesiense and T. b. gambiense) whilst diseases of domesticated livestock caused by trypanosomes are found in all continents except Australia (Hoare, 1972).

The trypanosome life cycle involves an alternation between two hosts. The vertebrate animal is the final host, whilst diverse haematophagous invertebrates represent the intermediate hosts or vectors which transmit the infection between vertebrates (Hoare, 1972). The vectors of mammalian trypanosomes are bloodsucking arthropods and the bloodstream form trypanosomes are ingested by the insect during the act of feeding. The trypanosomes then undergo a cycle of development culminating in the production of metacyclic trypanosomes which are transmitted to the new mammalian host. Development of the infective stages may occur in one of two sites within the vector; the hind gut or posterior station and the feeding apparatus or anterior

station. This has resulted in two methods of transmission: contaminative, where the trypanosomes infect the vertebrate host via infective faeces (e.g. T. cruzi in South America) and inoculative when the metacyclics are present in the saliva of the feeding insect (e.g. T. congolense). Successful transmission can only be effected when the parasite has completed its entire life cycle within the invertebrate host. This cyclical mode of transmission has been eliminated by some mammalian trypanosomes such as T. evansi where the parasite can be transferred directly from one mammal to another mechanically via biting flies. In contrast to cyclical transmission which may be effective for the lifetime of an infected vector, the ability to transmit trypanosomes mechanically is of short duration since it depends entirely on the length of survival of the bloodstream forms on the mouthparts of the insect.

Trypanosomes cyclically transmitted by tsetse flies which belong to the genus Glossina are found only in Africa and the infections caused by these parasites are known as the African trypanosomiases. However, each infection in a particular animal host by a particular trypanosome species can be regarded as a distinct disease entity (Mulligan, 1970; Losos and Ikede, 1972). The normal vertebrate hosts of these Trypanosoma species are wild mammals which unless otherwise stressed, generally do not suffer any pathogenic effects from the trypanosome infection. These animals act as reservoir hosts from which the tsetse flies acquire infections. Once infected, the tsetse flies themselves become reservoirs of the parasites and when cyclical development is completed, the tsetse flies then have the potential to

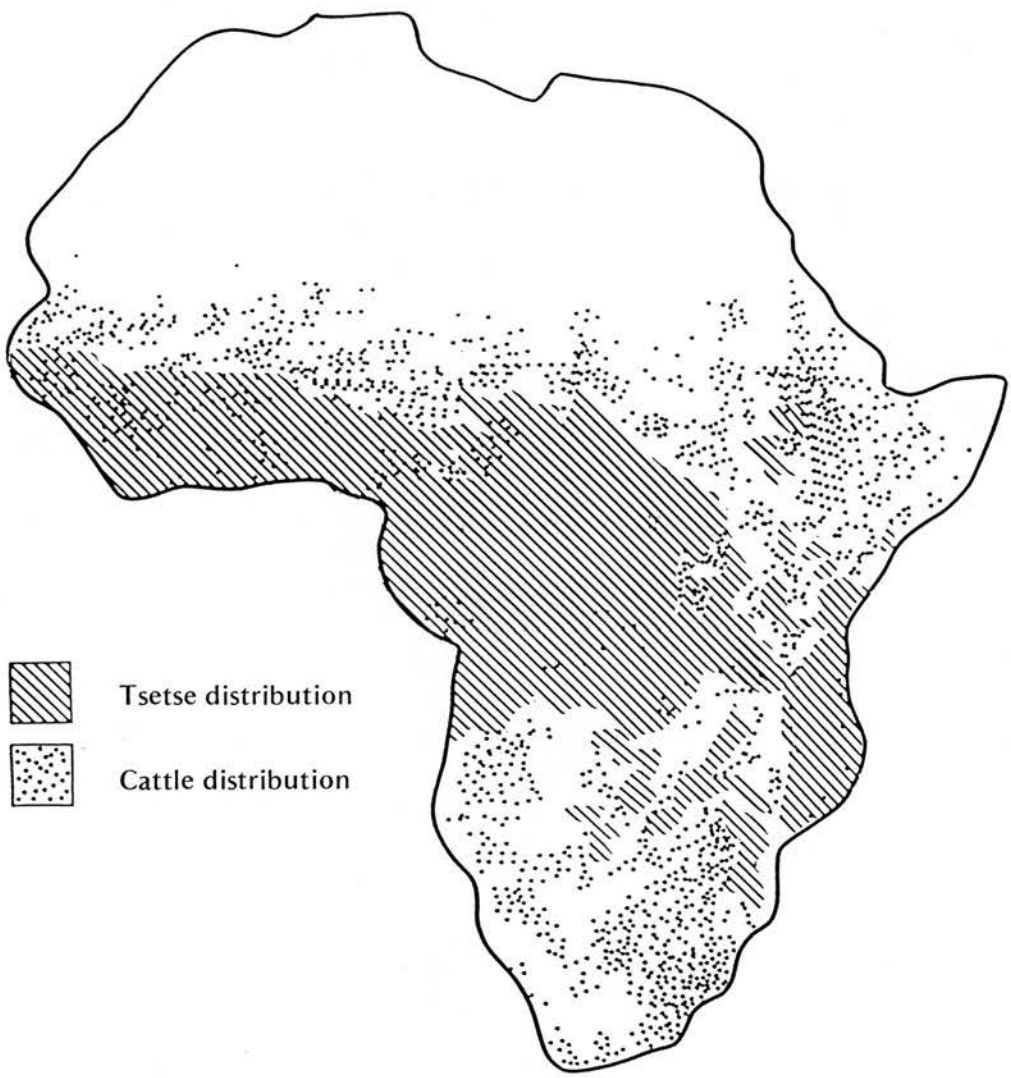
infect any animal from which it takes a blood meal (Jordan, 1986). When man or his domesticated animals, which are usually susceptible to the pathogenic effects of trypanosomes, intrude into this cycle the diseases sleeping sickness in man and nagana in domesticated livestock result.

Man is susceptible to two sub-species of Trypanosoma brucei: T. b. rhodesiense causes an acute form of sleeping sickness in East and Central Africa whilst T. b. gambiense causes a more chronic form in West Africa. However, typical forms of the two diseases are by no means universal with acute forms of T. b. gambiense sleeping sickness (Scott, 1970) and chronic forms of T. b. rhodesiense sleeping sickness (Apted, 1970) reported within respective distributions for both parasites.

Cattle, goats and pigs are the most important domesticated livestock affected by the tsetse borne trypanosomiases and the presence of tsetse completely precludes their maintenance in many areas of Africa (Figure 1.1). The pathogenic species involved are T. vivax and T. congolense in cattle and goats and T. simiae in pigs. T. brucei sub-species are generally regarded as of little pathogenic importance in domesticated livestock but may be important in the epidemiology of human trypanosomiasis since T. brucei rhodesiense has been isolated from cattle and the pig has been implicated as a reservoir host of T. b. gambiense (Onyango, Van Hoeve and de Raadt, 1966; Mehlitz, Zillmann, Scott and Godfrey, 1982). The animal trypanosomiases also affect human health in that losses in meat and milk production lead to protein deficiencies and malnutrition. It has been suggested that purely on medical grounds, the effects of animal

FIGURE 1.1

Approximate distribution of cattle and tsetse flies in Africa.



trypanosomiases are possibly a greater problem than human trypanosomiasis (Willet, 1970). Trypanosomiasis caused by T. vivax and T. congolense can result in death and abortion but more frequently, direct losses are due to chronic disease resulting in infertility, reduced growth and milk production. Indirect losses result from limitations in the use of exotic imported breeds and draught animals (Finelle, 1980).

Tsetse-transmitted animal trypanosomiases are one of the major constraints to socio-economic development in Africa. Tsetse flies infest approximately ten million km^2 representing 37% of the continent, 38 countries or half the habitable land (FAO/WHO/OIE, 1982). Approximately seven million km^2 of this area would be suitable for mixed agriculture and/or livestock development if freed from tsetse flies (MacLennan, 1980). It is estimated that about 30% of the 147 million cattle in the countries affected by tsetse are exposed to the disease (FAO/WHO/OIE, 1982). The situation with regard to sheep, goats, pigs, horses, donkeys and camels is probably similar but it is less well documented (Murray and Gray, 1984). The annual loss in meat production alone due to trypanosomiasis was valued at $5 billion US at 1963 prices (FAO/WHO/OIE, 1963). This excludes losses in milk and mixed agriculture where traction and manure play a vital role since animals provide 80% of traction power in Africa agriculture (McDowell, 1977).

A large part of the success of trypanosomes as parasites is their ability to undergo antigenic variation and thus evade the immune response of the host. Antigenic variation is a process whereby trypanosomes sequentially express a series of different surface

antigens called variable antigen types (VATs). Variable antigen completely covers the surface of the bloodstream form and the infective metacyclic trypanosomes. The surface coat is an electron dense layer 12-15 nm thick and consists of a monomolecular layer of glycoprotein called the variable surface glycoprotein (VSG). Each trypanosome expresses only one VSG and therefore one VAT at a time. However, the ability to change VAT is intrinsic in every dividing trypanosome since cloned parasites can give rise to many different VATs. Antigenic switching by trypanosomes is a process that does not require antibody (Doyle, Hirumi, Hirumi, Lupton and Cross, 1980; Luckins, Frame, Gray, Crowe and Ross, 1986) and appears to be a result of changes at the expression site within the trypanosome genome (Barry, 1986). The number of different VATs that a trypanosome may express in the bloodstream of the infected host is unknown, but experimental studies with T. equiperdum found at least 101 different VATs (Capbern, Giroud, Baltz and Mattern, 1977). Estimates based on analysis of the genome of one stock of T. brucei suggest that there are about 1,000 VSG genes per trypanosome nucleus although not all of these genes are expressed as VATs (Van der Ploeg, Valerio, De Lange, Bernards, Borst and Grosveld, 1982; Barry, 1986). It appears likely that both T. vivax and T. congolense bloodstream form trypanosomes are also capable of expressing several hundred VATs. Each trypanosome has the capacity to express a characteristic repertoire of VATs during the course of an infection. Cloned stocks of trypanosomes that express the same VAT repertoire belong to the same serodeme (Van Meirvenne, Janssens and Magnus, 1975a; Anon, 1978).

Early studies suggested that after cyclical development within

the tsetse fly each cloned stock reverted to a 'basic antigen' which was stable and characteristic for that stock (Broom and Brown, 1940; Gray, 1965a). In addition, certain antigenic types termed 'predominant antigens' tended to appear in a predictable manner during the early stages of infection of the mammalian host (Gray, 1965b). Initial attempts to serologically type trypanosomes were based either on basic strain antigens extruded from tsetse flies (Goedbloed, Ligthart, Minter, Wilson, Dar and Paris, 1973) or on 'predominant antigens' obtained from laboratory animal infections (Gray, 1975; Van Meirvenne, Magnus and Vervoort, 1977). Whilst these approaches were successful, particularly with T. brucei sub-group trypanosomes, there were inherent disadvantages in the limited availability of metacyclic trypanosomes and difficulties encountered due to antigenic variation when attempting to raise predominant antigens in laboratory hosts.

It is now evident that the metacyclic population for any given serodeme consists of a mixture of antigenic types although comprising only a fraction of the entire VAT repertoire (Le Ray, Barry and Vickerman, 1978; Barry, Crowe and Vickerman, 1983; Crowe, Barry, Luckins, Ross and Vickerman, 1983). In addition, the M-VATs of a particular serodeme are predictable in both their presence and prevalence in the metacyclic population (Nantulya, Doyle and Jenni, 1980; Akol and Murray, 1983) although there is evidence for one serodeme of T. b. rhodesiense that this predictability might not persist over repeated tsetse fly transmission between mammals (Barry et al, 1983). Sexual recombination between trypanosomes belonging to different serodemes has been identified (Tait, 1980; Jenni, Marti, Schweizer, Betschart, Le Page, Wells, Tait, Paindavoine, Pays and

Steinert, 1986) and this could lead to further instability of the M-VAT repertoire. Nevertheless, characterization of M-VAT repertoires with their limited number of VATs and stage-specific expression appears to be the most suitable approach to serodeme analysis.

The number of metacyclic forms produced from tsetse flies infected with T. brucei has been shown to be as high as 40,000 although the average number produced is much lower at around 3,200 (Harley, Cunningham and Van Hoeve, 1966). In contrast, T. congolense infected Glossina produce much fewer metacyclics varying from between three and 350 per infective feed (Harley and Wilson, 1968) thus limiting the availability of this stage for serological studies of T. congolense M-VAT repertoires.

There have been several attempts at overcoming the low number of metacyclics and their poor infectivity and virulence for laboratory animals. Efforts to raise bloodstream form trypanosomes expressing M-VATs by feeding tsetse flies on immunosuppressed mice had little success with T. congolense in comparison with T. brucei (Schlappi and Jenni, 1977). There was very little reduction in the prepatent period in immunosuppressed mice compared to normal mice. When sufficient numbers of trypanosomes were available for serological analysis, antigenic variation had occurred and the parasites collected from the blood of the host were antigenically unrelated to the metacyclic trypanosomes inoculated by the tsetse flies.

A novel approach to examining antigenic relationships between stocks was taken using trypanosomes developing in local reactions in rabbit skin at the sites of bites by infected tsetse flies as antigens in immunofluorescence assays (Luckins and Gray, 1979a). This

approach was partially successful, one disadvantage was that not all trypanosome stocks produced local skin reactions in rabbits. The feasibility of using local skin reactions to characterize metacyclic populations was also examined by Dwinger, Murray and Moloo (1987). The rationale behind their approach was that animals which are infected with tsetse transmitted trypanosomes and then drug treated are immune to challenge with homologous trypanosomes; they do not develop any detectable skin reaction at the site of challenge and they do not become infected. However, if they are challenged by an antigenically unrelated (heterologous) stock of trypanosomes, they develop chancres and become infected (Akol and Murray, 1983). Although this approach was successful, problems were encountered when attempts were made to induce immunity to more than one serodeme either by simultaneous infection or superinfection.

Research on T. congolense has benefited from the recent advances in trypanosome in vitro culture techniques and in particular the development of an insect form culture system (Gray, Cunningham, Gardiner, Taylor and Luckins, 1981; Gray, Ross, Taylor and Luckins, 1984; Gray, Hirumi and Gardiner, 1987). Using this system, large numbers of metacyclics can be obtained on a regular basis. In addition, the metacyclics are antigenically stable over long periods in culture (Luckins et al, 1986). Therefore, direct experimentation on metacyclic trypanosomes and their VAT repertoires using in vitro-derived metacyclic forms is now possible.

The ability to identify and enumerate trypanosome serodemes circulating in defined areas is important, particularly in relation to studies on the epidemiology of trypanosomiasis. Also, by examining

the distribution of serodemes the feasibility of control measures such as immunoprophylaxis could be accurately assessed. For example, in areas where there are few serodemes and the serodeme distribution remains stable over a period of time due to an isolated focus of the disease, a rational approach to chemotherapy involving the acquisition of immunity could possibly be employed (Wilson, 1971; Wilson, Paris and Dar, 1975; Wilson, Paris, Luckins, Dar and Gray, 1976; Masake, Nantulya, Musoke, Moloo and Nguli, 1987).

There have been no attempts to characterize M-VATs of T. congolense as a means of establishing the antigenic relationships between different isolates. The work described in this thesis was undertaken to identify and enumerate T. congolense serodemes, using metacyclic trypanosomes, from stocks of trypanosomes isolated from domestic dogs in a 320 hectare (ha) area around Kakumbi, Chipata District, Zambia in 1981. Isolates of T. congolense were cloned and then used to initiate insect form in vitro cultures producing metacyclic trypanosomes. Monoclonal antibodies were prepared against one stock to define its M-VAT repertoire and to determine if similar M-VATs were present in other stocks. Thereafter, serological assays were developed using the cultured metacyclic trypanosomes to examine their relationships and to provide a possible means to monitor the diversity and distribution of T. congolense serodemes.

CHAPTER TWO

REVIEW OF THE LITERATURE

2.1 CLASSIFICATION

Trypanosomes are classified in the genus Trypanosoma Gruby, 1843 and are members of the sub-kingdom Protozoa, order Kinetoplastida (Honigberg, 1963 emend Vickerman, 1976) family, Trypanosomatidae (Doflein, 1901 emend Grobben, 1905). The systematic classification is shown in Table 2.1.

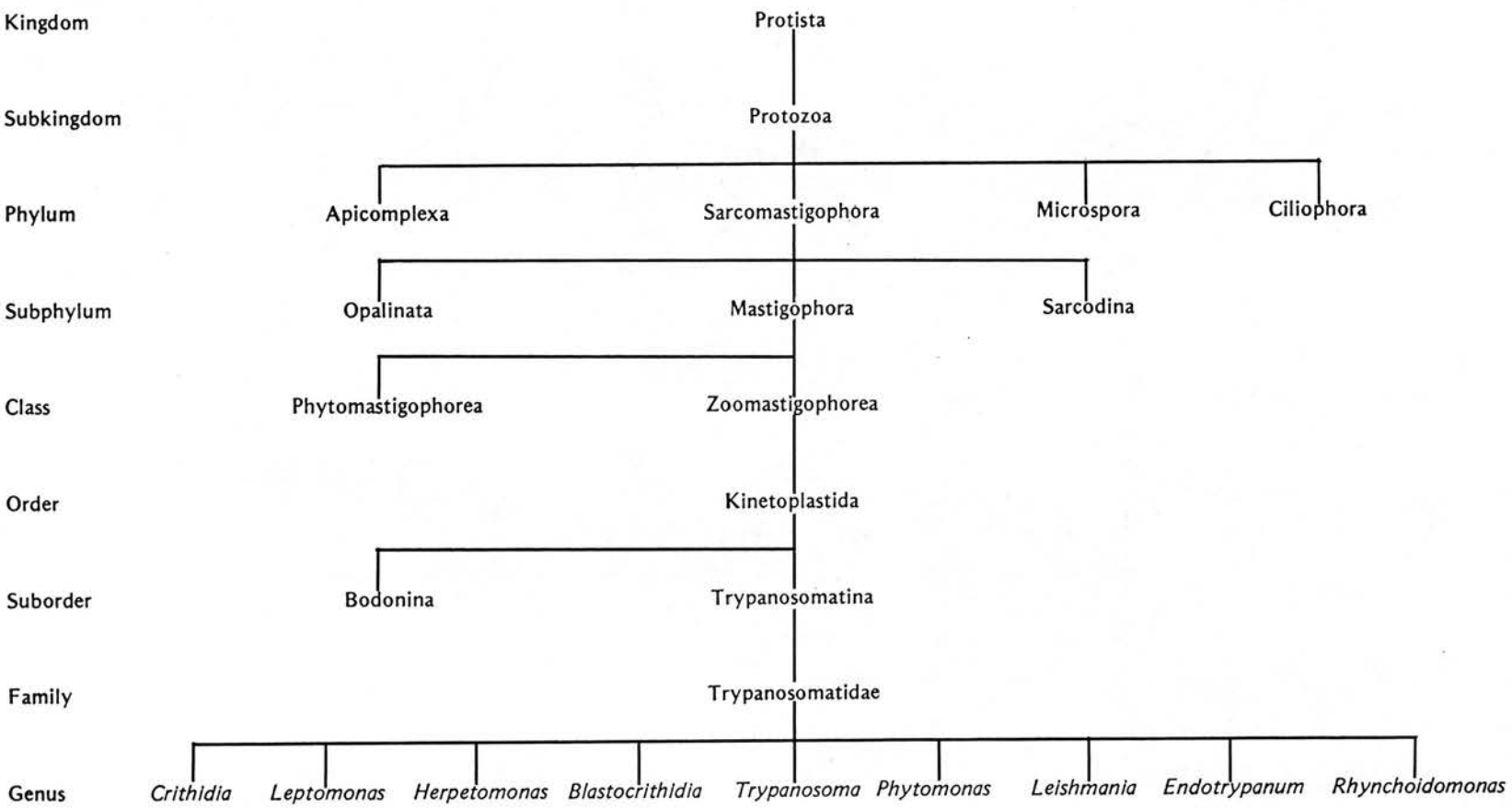
2.1.1 Genus Trypanosoma Gruby, 1843

The genus Trypanosoma consists of digenetic flagellates which parasitize the blood of vertebrates and the gut of leeches or arthropods; trypomastigote and epimastigote stages occur in nearly all trypanosome life cycles but amastigotes and rarely promastigotes may also be present (Hoare, 1972). A haematozoic trypomastigote form is common to all species in the vertebrate host. The form is basically lanceolate in shape, its body having the form of an elongated flattened blade which is elliptical or oval in transverse section while its ends taper to a point. The type species is T. rotatorium (Mayer, 1843) which is a parasite of frogs.

The mammalian trypanosomes have been divided into sub-genera on the basis of the pattern of development of the parasites in the insect host (Hoare, 1964). The sub-genera are further divided into two sections, Stercoraria and Salivaria which are based on the site of production of metacyclic trypanosomes in the insect host and the subsequent method of infection of the mammalian host.

TABLE 2.1

Classification of Kinetoplastida.



(From Molyneux and Ashford, 1983)

The section Stercoraria is comprised of species whose developmental cycle in the insect host is completed in the hindgut: metacyclics are present in the faeces and transmission is contaminative when vector faeces are deposited on abraded skin or mucous membranes of the vertebrate host. There is only one species of pathogenic importance within this section, Trypanosoma (Schizotrypanum) cruzi the causative agent of Chagas' disease or American trypanosomiasis in Central or South America. Two non-pathogenic species T (Megatrypanum) theileri and T. M. melophagium may be of some importance in the diagnosis of trypanosome infections since they have a cosmopolitan distribution and are often found in mixed infections with pathogenic species (Hoare, 1972).

The developmental cycle of trypanosomes within the section Salivaria is completed in the mouthparts or salivary glands of the invertebrate host so that the metacyclics are present in the saliva. Transmission is by inoculative means during the course of feeding. The trypanosomes within this section are classified into three sub-genera according to their morphology within the mammalian host and whether developmental sites in the insect vector include the proboscis only (subgenus Duttonella), midgut and proboscis (subgenus Nannomonas) or midgut and salivary glands (subgenus Trypanozoon). In one species, T. evansi, there is no cycle of development in the invertebrate host which acts purely as a mechanical transmitter (Vickerman, 1976). A related species, T. equiperdum relies on coitus for transmission and has therefore eliminated entirely the need for a second host.

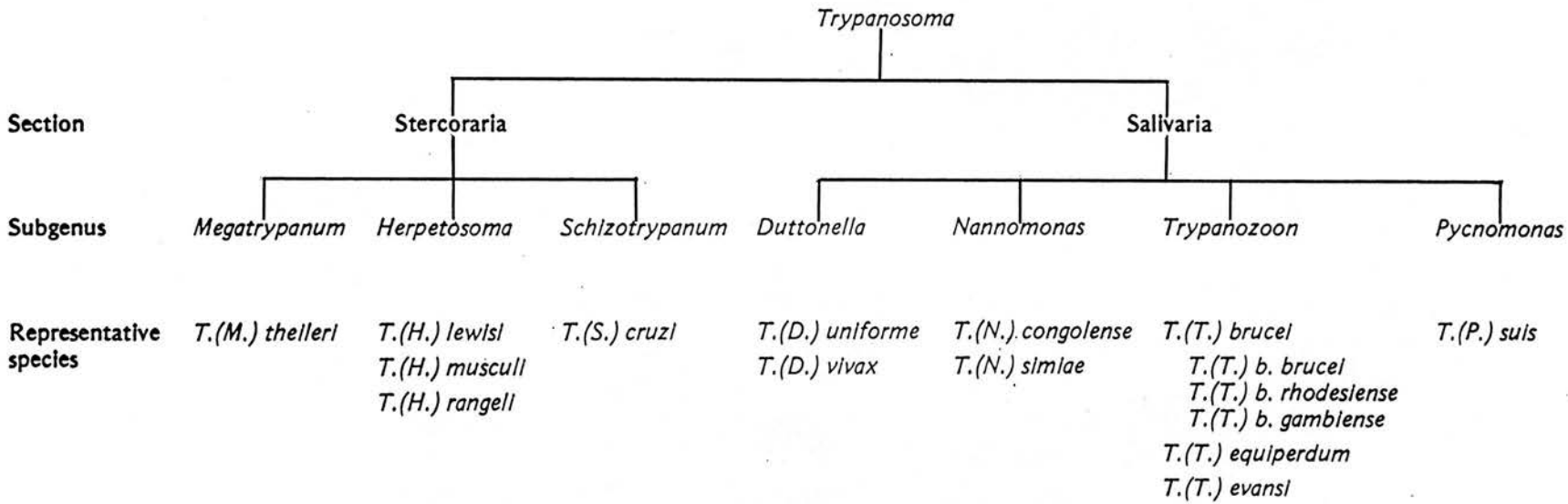
2.1.2 Speciation of trypanosomes within the section Salivaria

Table 2.2 shows a recent classification of mammalian trypanosomes based on the recommendations of Hoare (1972). Despite recent developments in recombinant DNA technology (Majiwa, Hamers, Van Meirvenne and Matthyssens, 1986) and isoenzyme characterization (Godfrey, 1977) the criteria for classifying trypanosomes to the species level are still assessed on the basis of morphology, host specificity and pathogenicity as used by Hoare (1972). Here it was argued that on the basis of integradation of behavioural characteristics each sub-genus in the section Salivaria should be regarded as monospecific but for practical purposes the previously recognized and long accepted speciation in the two sub-genera Duttonella and Nannomonas were retained. However, in the sub-genus Trypanozoon, Hoare (1972) recognises three species; T. evansi, T. equiperdum and T. brucei with three subspecies T. brucei brucei, T. b. rhodesiense and T. b. gambiense which were previously recognized as separate species.

It has been suggested that each salivarian sub-genus be regarded as monospecific with subspeciation being allowed for on the basis of behaviour in hosts such as man and domesticated animals (Anon, 1979). Therefore, a salivarian species has been defined as an assemblage of organisms which can be distinguished from other species by one or more stable discontinous morphological characters while a salivarian subspecies has been defined as an assemblage of organisms within a species which cannot be separated from each other by morphological characters but only by other stable characters (Anon, 1979). This is obviously inadequate and it appears that with the advent of molecular

TABLE 2.2

Classification of the genus *Trypanosoma*.



(From Molyneux and Ashford, 1983)

biological techniques, analysis of trypanosome DNA will provide a more accurate method of speciation.

2.1.3 Morphological identification of salivarian species

The speciation of salivarian trypanosomes is based partly on the morphological characteristics of the trypomastigote bloodstream form. The diagnostic features used for species identification are, total length, position and size of the kinetoplast, presence or absence of free flagellum and the extent of convolution of the undulating membrane (Hoare, 1972). These features are best observed in Giemsa-stained smears when relative positions of the kinetoplast and nucleus can be assessed. The morphological features of the different salivarian species are described by Hoare (1970, 1972) and Vickerman (1972).

2.1.4 Subgenus Nannomonas Hoare, 1964

Trypanosomes of the subgenus Nannomonas are represented by relatively small parasites measuring between 8-24 µm in total length. They are typically without a free flagellum throughout all stages of development although in some individuals a small free portion is visible. The kinetoplast is of medium size, being much smaller than in Duttonella but larger than in Trypanozoon while its position in the body is subterminal and marginal. The undulating membrane is inconspicuous in T. congolense but may be well developed in T. simiae (Hoare, 1972).

2.1.4.1 Trypanosoma (Nannomonas) congolense (Broden, 1904)

Broden (1904) discovered in the blood of sheep and a donkey at Leopoldville (Congo), small trypanosomes without a free flagellum

which differed from those recorded previously. This new trypanosome species was called Trypanosoma congolense and is the type species of the subgenus Nannomonas. The morphological and biological diversity shown by T. congolense has given rise to many synonyms and much confusion. Synonyms include T. dimorphon (Laveran and Mesnil, 1904); T. nanum (Laveran, 1905); T. confusum (Montgomery and Kinghorn, 1909); T. pecorum (Bruce, Hamerton, Bateman and Mackie, 1910); T. montgomeryi (Laveran, 1909) and others reviewed by Hoare (1972).

The problems of classification based on morphological characteristics are highlighted by the change in status of T. dimorphon which is now regarded purely as a synonym of T. congolense. T. dimorphon was the name given to a dimorphic trypanosome isolated from the blood of a horse in The Gambia. It was differentiated from T. congolense on the basis of its greater mean length and the proportions of long, short and intermediate forms. Hoare (1970) suggests that the first description of T. dimorphon was inadequate, and the name was frequently misused and trypanosomes of both types were for many years cited indiscriminately as T. congolense and/or T. dimorphon.

In a morphological and biometrical revision of T. dimorphon and T. congolense based on the original material studied by Laveran and Mesnil (1904), T. dimorphon was restored to the status of a distinct species (Hoare, 1959). This decision was based on statistical analysis on the mean lengths of the organisms which showed a clear difference (T. congolense 12.2-14.4 μm compared to T. dimorphon 15.3-17.6 μm). However, T. congolense stocks were discovered which had mean lengths between those of T. congolense and T. dimorphon (Godfrey, 1960; Fairbairn, 1962).

In another study of the original T dimorphon, Huisenga (1969) found that both T. congolense and T. dimorphon had the same mean lengths when measured exclusively in the initial stages of division implying that there was no justification for distinguishing subspecies or types based on their mean lengths. Hoare (1972) interpreted this finding as showing that in all stocks of T. congolense divisions are restricted to individuals of the same mean lengths and that these trypanosomes then differentiate into adult trypanosomal populations conforming to congolense, intermediate or dimorphon types (Godfrey, 1960; Fairbairn, 1962). These three morphological forms have been found at different stages of infections initiated with a single organism (Nantulya, Doyle and Jenni, 1978a). The morphological appearance of cloned trypanosomes in mice was found to change throughout the course of a parasitaemic wave. Five days post-infection, intermediate stages were detected. At the parasitaemic peak, seven to eight days post-infection, the trypanosome population was highly pleomorphic with long forms predominating and the overall picture was that attributable to a dimorphon type of T. congolense. Rapid syringe passage at three to four day intervals resulted in the trypanosome population transforming to a short congolense type.

The question as to whether different trypanosome species or subspecies comprise T. congolense is therefore an old one. Evidence has been presented to show diversity in T. congolense with regard to clinical differences in the disease they cause (Godfrey, 1961) and isoenzyme polymorphism among different isolates (Young and Godfrey, 1983; Gashumba, 1986; Knowles, Betschart, Kukla, Scott and Majiwa, 1988). There is now also evidence of genetic diversity in

trypanosomes classified as T. congolense (Knowles et al, 1988; Majiwa et al, 1986).

2.1.4.2 Trypanosoma (Nannomonas) simiae Bruce et al, 1912

Trypanosoma simiae was the name given by Bruce et al (1912) to a trypanosome isolated from a monkey infected from wild-caught tsetse flies. Although named after the host it was first derived from, T. simiae is primarily a parasite of pigs and its extreme virulence and pathogenicity for this species has no parallel amongst the mammalian trypanosomes (Hoare, 1972). T. simiae can be distinguished from T. congolense by its greater mean length (15.3 μm - 19.7 μm) compared to that of T. congolense (12 μm - 15.7 μm), its marked pathogenicity for the pig and lack of infectivity for cattle, mice and rats and also the fact that it is refractory to most chemotherapeutic drugs available (MacKenzie and Boyt, 1969; Hoare, 1970, 1972; Stephen, 1986).

There have been doubts about the validity of T. simiae as a distinct species on the basis of accepted criteria. The length of T. simiae could be influenced by host species and one stock when passaged in sheep was found to have a mean length which fell within the T. congolense range (Roberts, 1971). The species of Glossina responsible for transmitting T. simiae can also affect the degree of pathogenicity in pigs (Janssen and Wijers, 1974; Agu, 1984). When a stock of T. simiae was transmitted by G. brevipalpis it was more virulent to pigs than the same stock when transmitted by G. pallidipes.

Recently, species-specific DNA probes based on repetitive DNA (satellite DNA) hybridization have been developed which can differentiate between T. congolense and T. simiae (Majiwa and Webster,

1987; Gibson, Dukes and Gashumba, 1988). These probes have been used in a field survey in the Gambia to identify trypanosomes in tsetse fly midguts (McNamara, Dukes, Snow and Gibson, 1989).

2.2 VECTORS OF AFRICAN SALIVARIAN TRYPANOSOMES

The transmission of the African salivarian trypanosomes is effected by Glossina spp. which are commonly called tsetse flies. As early as 1857, Livingstone linked the tsetse fly with "nagana" and later nagana was shown to be caused by trypanosomes which were transmitted to the mammal via tsetse flies (Bruce, 1895). Kleine (1909) first demonstrated the cyclical development of T. brucei within Glossina fuscipes and thereafter many reports were made of cyclical development of various Trypanosoma species within numerous species of Glossina (reviewed by: Hoare, 1972; Stephen, 1986).

Tsetse flies are larviparous, haematophagous dipterans; both the male and female feed only on blood and are therefore equally important in the transmission of trypanosomiasis. Twenty two species and 14 subspecies are generally recognized and although the flies are found exclusively in Africa between latitudes 15^{o}N and 28^{o}S, many species have a limited local distribution. There are three major groups, fusca, palpalis and morsitans. Members of the fusca group tend to be associated with more humid environments and forest habitats whilst members of the palpalis group are generally confined to waterside habitats. The morsitans group are usually found in areas of drier savanna vegetation. In general, savanna and forest species are more efficient vectors of trypanosomes than riverine species but because

of their proximity to grazing areas, savanna and riverine species present the major threat to livestock (Jordan, 1986).

2.3 LIFE CYCLE OF TRYPANOSOMA CONGOLENSE

2.3.1 Development of T. congolense within the insect vector

When a receptive species of Glossina feeds on a mammal harbouring T. congolense, the trypomastigotes pass into the midgut of the fly in the blood meal. Here, they begin a cycle of development within the lumen of the peritrophic membrane particularly in the terminal third region of the membrane (Stephen, 1986). At this site the trypanosomes change their morphology and physiology and become elongated trypomastigote forms or procyclics in which the kinetoplast is located close to the nucleus (Figure 2.1) and the mitochondrion expands into a branched network of discoid rather than tubular cristae. Procyclic development is associated with a switch from the utilization of glucose to the utilization of proline as a principle energy source which is more suited to the insect vector. The flagellates multiply actively and may persist in this area for some time. However, as with T. brucei (Ellis and Evans, 1977) the trypanosomes are able to penetrate the fully formed peritrophic membrane in the central region of the midgut thus gaining access to the ectoperitrophic space without having to pass around the open end of the peritrophic membrane in the hindgut where the environment is hostile to the trypanosomes (Evans, Ellis and Stamford, 1979). However, it has also been reported that the procyclic trypomastigotes invade the ectoperitrophic space via the open end of the peritrophic membrane at

FIGURE 2.1

Stages in the life cycle of *Trypanosoma congolense*.

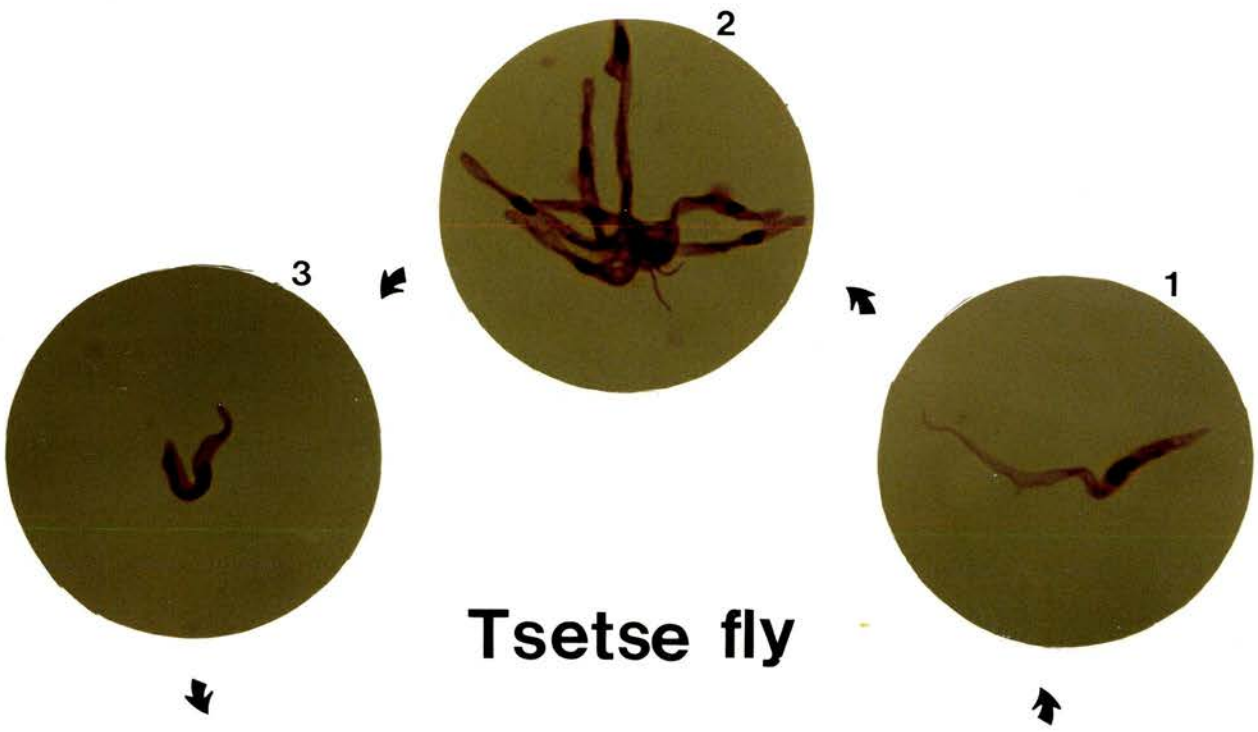
1 Procyclic

2 Epimastigote

3 Metacyclic

4 Local skin reaction (or chancre)

5 Bloodstream


2
3
1
Tsetse fly


Mammal
4
5

the hindgut and then migrate forward in the ectoperitrophic space between the membrane and the cells lining the midgut wall (Gordon, 1957; Yorke, Murgatroyd and Hawking, 1933). Behind the peritrophic membrane, the trypanosomes are protected from lysins present in the bloodmeal (Gordon, 1957).

The procyclic trypanosomes then migrate forwards until they reach the cardia (proventriculus) where they pass from the ectoperitrophic space of the cardia into its endoperitrophic space by penetrating the least resistant portion of the membrane namely, the fluid or semi-fluid part at or near the points where it is being secreted (Gordon, 1957; Hoare, 1970). Having gained an entrance into the inner lumen of the cardia, the trypanosomes continue to migrate forwards, first into the oesophagus and pharynx and then into the proboscis where T. congolense completes its development.

The long trypomastigotes attach and transform to epimastigotes which are characterized by an anterior positioning of the kinetoplast in relation to the nucleus (Figure 2.1). The epimastigote forms attach preferentially in the proximal third region of the tsetse labrum (Clarke, 1965; Thevenaz and Hecker, 1980) and the attachment is by means of hemidesmosomes between trypanosome flagellum and the insect cuticle (Hommel and Robertson, 1976). Contact with the cuticle alone appears not always to induce the flagellum to form hemidesmosomes, but contact with other flagella and/or existing hemidesmosomes seems to favour the formation of new junctions between flagella and cuticle. Attachment structures in T. congolense are probably necessary to prevent the developing stages being evacuated

due to strong mechanical forces caused by the rapid flow of fluids in the labrum (Thevenaz and Hecker, 1980).

The colonization of the labrum by epimastigotes occurs in a region bearing mechanoreceptors responsible for detecting the rate of blood flow (Clarke, 1965; Rice, Galun and Margalit, 1973; Molyneux, Lavin and Elce, 1979). Impairment of this feedback mechanism in flies with labral infections could confer a distinct biological advantage on the parasite with increased chances of transmission to the mammalian host.

It was thought that the transformation of the T. congolense epimastigote forms to the infective metacyclics took place only in the tsetse fly hypopharynx (Wenyon, 1926; Hoare, 1972). However, in a more recent study, trypanosomes covered by a surface coat were also found attached to the proximal part of the food canal indicating that the transformation to metacyclic forms is not strictly limited to the hypopharynx (Thevenaz and Hecker, 1980). These attached and coated forms are probably equivalent to the nascent metacyclics of T. brucei which although attached to the salivary gland epithelium possess a glycoprotein surface coat (Vickerman, 1985; Tetley, Turner, Barry, Crowe and Vickerman, 1987). Fully matured metacyclic forms of T. congolense possess the surface coat but lie free in the lumen of the hypopharynx and have never been seen to divide.

This entire life cycle within the vector, from ingestion of bloodstream forms to the production of infective metacyclic forms, was held to take between 19 and 53 days to complete (Hoare, 1970). However, a more recent report suggests that the life cycle can take between seven and 40 days to complete with 45% of laboratory reared

flies becoming infective by day 12 post-infection and 76% by day 18 post-infection (Nantulya and Doyle, 1978b*). There is no clear evidence on what factor or factors influence the duration of the life cycle but some suggestions have included, the stock of trypanosome, species of host on which the fly feeds, species of fly, and the morphology and number of trypanosomes ingested by the fly (Corson, 1935; Wijers and Willet, 1960; Nantulya et al, 1978b).

There have been several investigations into factors which influence trypanosome infection rates in tsetse flies. Rates of infection could be raised from 15.5% to 35% by raising the temperature at which Glossina pupae were incubated to 28°C (Godfrey, 1958, 1959). However, when the maintenance temperature for adult G. m. morsitans was raised from 26°C to 31°C, there was no effect on the tsetse flies' susceptibility to T. congolense (Dipeolu, 1975). Infection rates vary between species. When G. morsitans, G. pallidipes and G. fuscipes were compared as vectors of T. congolense, their infection rates were found to be 11.6%, 13.2% and 2.9% respectively (Harley and Wilson, 1968) and several studies have demonstrated the complete inability of G. palpalis to act as a vector for T. congolense (Duke, 1923; Godfrey, 1961; Willet, McMahon, Ashcroft and Baker, 1964).

The influence of the level of parasitaemia in the infective blood meal on subsequent fly infection rates was investigated but found to have no effect (Dipeolu, 1975). However, it has subsequently been shown that teneral G. morsitans fed on mice at peak parasitaemia with T. congolense had higher infection rates with those fed before or after peak parasitaemia (Nantulya, Doyle and Jenni, 1978b).

* Nantulya, Doyle and Jenni, 1978b.

Infection rates of 7.1%, 22.4% and 6.6% were obtained for flies fed on log phase of a rising parasitaemia, peak parasitaemia and following phase of a parasitaemic wave respectively.

The mammalian host species from which the flies take the infected bloodmeal is also thought to have an influence on Glossina infection rates: G. m. morsitans were found to have higher infection rates when fed on goats or rabbits compared to those fed on rats or mice (Moloo, 1981).

2.3.2 Development of T. congolense within the mammalian host

T. congolense infects the mammalian host via the discharged saliva of the Glossina vector during feeding. Of all the stages of development within the insect vector, only the metacyclic trypomastigote (Figure 2.1) is known to initiate infection. Although T. congolense had been regarded as a strict haematic parasite (Losos and Ikede, 1972; Losos, Paris, Wilson and Dar, 1973), the local skin reaction or chancre which develops following trypanosome development in the dermal collagen of the mammalian host was shown to be an exception to this haematic distribution.

The development of local skin reactions in infections with T. congolense in domesticated animals was first reported in cattle and sheep at sites bitten by experimentally infected G. morsitans and G. tachinoides and also wild caught G. morsitans (Roberts, Gray and Gray, 1969). The reactions appeared four to 12 days before patent parasitaemia as determined by examination of wet blood films. Exudate from chancres which were examined microscopically revealed trypanosomes morphologically indistinguishable from bloodstream forms and

also organisms which were longer and more slender than either the metacyclic or the bloodstream forms and resembled trypanosomes found in the midgut and proboscis of the infected tsetse fly.

Chancres have been reported in several host species including goats (Emery and Moloo, 1980; Dwinger, 1985), sheep (Uilenberg, Maillot and Giret, 1973), cattle (Roberts et al, 1969; Akol and Murray, 1982) and rabbits (Gray and Luckins, 1980a; Luckins and Gray, 1983). The size and severity of the chancre varies with the strain of the trypanosome and the species and status of the host (Dwinger, 1985). In smaller laboratory animals such as mice, rats and guinea pigs chancres do not form (Emery and Moloo, 1980). In these animals, trypanosomes appear to be deposited subcutaneously and rapidly enter the bloodstream. This suggests that the chancre is associated with skin thickness and a physiological response of the skin which restricts the immediate spread of the trypanosomes and allows parasite multiplication (Emery and Moloo, 1980). Early reports that the local skin reaction was necessary for metacyclic trypanosomes to adapt to the mammalian host and that it provided a localized site for parasite multiplication are now known not to be true since some stocks of T. congolense consistently fail to produce local skin reactions (Gray and Luckins, 1980a) but do give rise to systemic infections. Also, metacyclic trypanosomes cause infections when inoculated directly into the host's bloodstream.

Losos and Ikede (1972) and Losos (1979) have suggested that the chancre is an experimental phenomenon since there have been no reports of its natural occurrence in the field. However, among the factors which could contribute to the lack of field observations on

chancre development are the interference phenomenon where in concurrent infections chancre induction is prohibited when animals are superinfected by unrelated serodemes (Luckins and Gray, 1983; Luckins, Rae and Gray, 1983). Also, chancre formation occurs largely within the first 10-15 days of infection in immunologically naive hosts and experimentally induced chancres are normally examined on a shaven area of the mammalian host's skin.

Histologically, the skin at the site of the chancre undergoes marked changes in cellularity and structure. Generally, between approximately day eight and day 15 post-infection there is a progressive disorganization and degeneration of dermal collagen during which a large cellular infiltrate occurs. The marked inflammation subsides between days 14 and 21 post-infection. In cattle, the cellular infiltrate associated with T. congolense consisted principally of lymphocytes and macrophages (Gray and Luckins, 1980b) although Akol and Murray (1982) described an initial inflammatory reaction containing numerous polymorphonuclear leucocytes (PMN) which were subsequently replaced by lymphocytes and plasma cells. The differences in the cellular infiltrates described in these two studies could be due to the fact that different stocks were used as well as the timing of the skin biopsy. T. congolense infections in goats have been shown to produce an initial influx of PMN in the skin followed by a substantial infiltration of lymphocytes and macrophages; plasma cells remained in large numbers at the skin reaction during its decline (Emery and Moloo, 1981). The initial cellular infiltrate is probably a response to chemotactic factors (Cook, 1980) as well as other factors generated by the parasites which increase

vascular permeability (Tizard and Holmes, 1977). Subsequent infiltration of plasma cells is probably associated with an immune response to the parasite surface antigens although the exact nature of this response is as yet unknown.

In quantitative histological studies of trypanosome development within the chancres induced by rabbits, sheep and calves, Gray and Luckins (1980b) showed that in rabbits at day seven post-infection, numerous trypanosomes were associated with or were inside collagen bundles in the deep dermis. More trypanosomes were present at day nine post-infection but by day 11, the number was decreasing. A similar trend was seen in reactions from calves at eight, ten and 12 days post-infection.

It is not known what causes trypanosomes in the collagen to leave their site of development but the disappearance of T. congolense from the lesion could be related to biological changes associated with their differentiation to bloodstream forms.

The route of dissemination of trypanosomes from the chancre is probably via the local lymphatic drainage system. Trypanosomes have been observed in the sinuses of lymph nodes of a calf and sheep ten days after cyclical infection when they were absent in the blood (Luckins and Gray, 1979b). In contrast, trypanosomes have been reported to be scarce or absent in the lymph of animals with T. congolense infections initiated by bloodstream forms (Ssenyonga and Adam, 1975; Tizard, Hay and Wilkie, 1978).

There is evidence that the distribution of T. congolense in the circulatory system is not uniform. There are often more trypanosomes in the capillaries than in arteries and veins and the

superficial vessels contain more trypanosomes when the animal is cool. A rise in external temperature or treatment with trypanocidal drugs increase temporarily the numbers of trypanosomes in the veins (Hornby and Bailey, 1931). However, the viability of these trypanosomes was not examined and it is possible, certainly after drug treatment that the trypanosomes detected could be dying.

Although there is a great deal of evidence that T. congolense resides within the lumen of blood vessels (Fiennes, 1946; Hornby and Bailey, 1931; Losos and Ikede, 1972; Losos, Paris et al, 1973; Ssenyonga and Adam, 1975), it has also been shown that in the microvasculature trypanosomes adhered to the vessel walls via their anterior ends (Banks, 1978). The bloodstream form parasites were shown to localize in clusters within the smaller vessels of the mesentery. No morphologically distinct features were noted at these attachment sites.

The preference of T. congolense for specific locations within the microcirculation was suggested from findings in other studies where more organisms were found in skeletal muscles, brain and myocardium than in the spleen and liver (Hornby and Bailey, 1931; Losos et al, 1973). The reasons for T. congolense locating in specific blood vessels have not been determined but could be associated with differences in blood pressure, blood flow patterns, endothelial cells or surfaces covering the lumen surfaces (Banks, 1978).

2.4 ANTIGENIC VARIATION

The success of trypanosomes as parasites is due in part to their ability to undergo antigenic variation (Ritz, 1916; Gray, 1965a).

This is a process whereby trypanosomes sequentially express a series of different surface antigens. These antigens are capable of inducing protective immunity and the immune response to each variant although rapid and highly efficient in destroying any trypanosomes that possess that particular antigen is invariably too late to affect trypanosomes which have altered their antigenic identity (Murray, Barry, Morrison, Williams, Hirumi and Rovis, 1979). Although, antigenic variation probably occurs when the trypanosomes start dividing in the local skin reaction, it is in the systemic phase, as bloodstream forms, that the trypanosomes undergo extensive antigenic variation. Thus, in the infected host parasitaemia rises and falls in waves with each peak representing the expression of a major VAT (homotype) although minor VATs (heterotypes) are also present and any one of these heterotypes may become the homotype of a subsequent population (Vickerman, 1978). Hence, a combination of a subsequent variation on the part of the trypanosome and immunological destruction on the part of the host appears to be responsible for the parasitaemic wave observed in trypanosome infections (Esser, Schoenbechler and Gingrich, 1982).

Antigenic variation in all the African trypanosomes is a consequence of changes in the composition of a surface coat which covers the entire surface of the trypanosome including the flagellum when it is in the bloodstream of the mammalian host (Vickerman, 1969; Vickerman and Luckins, 1969). The coat is lost when the trypanosome embarks on cyclical development within the tsetse fly and is reacquired when the trypanosome differentiates to the metacyclic form in preparation for inoculation into the mammalian host (Vickerman,

1969; Steiger, 1973; Vickerman, Barry, Hajduk and Tetley, 1980). The surface coat is composed of about 10^7 molecules of VSG and on each trypanosome only one VSG is expressed at a time (Cross, 1975) although two different VSG's have been observed on a single trypanosome during the switching process (Esser and Schoenbechler, 1985).

Selective pressure may operate to conserve perhaps only a general structure of the VSG molecule within the region involving binding to the cell membrane and possibly some areas associated with the intracellular processing of the newly synthesized VSG molecule (Barry, 1986). Indeed, pressure is operating for diversity in the regions of the molecule exposed to the host. The VSG molecule has two domains, the amino domain consisting of about 400 amino acids and the carboxy domain with 100 amino acids (Johnson and Cross, 1979). Within the amino domain there is no conservation except for some cysteine residues (Olafson, Clarke, Keilland, Pearson, Barbet and McGuire, 1984; Barbet, 1985). Analysis using monoclonal antibodies has revealed several antigenic determinants in this domain although only one determinant, which is formed as a result of protein folding, appears to be exposed to the host on the living trypanosome (Miller, Allan and Turner, 1984a).

The number of different VATs able to be expressed by a single trypanosome is unknown but direct cloning of trypanosomes has shown that in one stock of T. equiperdum there are at least 101 different VATs (Capbern et al, 1977). Estimates based on gene cloning and DNA hybridization suggests that each trypanosome contains 1,000-2,000 VSG genes (Van der Ploeg et al, 1982). Many genes are so similar that they may not encode antigenically distinguishable VSG's but the

genetic repertoire can be rapidly expanded by several mechanisms including segmental gene conversion which can result in the immediate expression of a novel VSG for which no unique gene pre-existed in the genome (Pays, Van Assel, Laurent, Darville, Vervoort, Van Meirvenne and Steinert, 1983a; Pays, Delauw, Van Assel, Laurent, Vervoort, Van Meirvenne and Steinert, 1983b).

The frequency at which the switching of one antigenic type to another type occurs was thought to be between 10^{-4} and 10^{-5} changes per cell generation (Van Meirvenne et al, 1975a; Doyle, 1977). However, these figures only represent the number of switched trypanosomes in the population at a particular time and do not represent the switching frequency. Lamont, Tucker and Cross (1986), calculated switching frequencies that ranged from 1.4×10^{-7} to 3.5×10^{-6}. Their calculations considered the growth rates of the newly expressed antigenic types, the proportion of switched trypanosomes and the number of generations from the original antigenically homogeneous population.

Switching from one VSG to another is independent of antibody pressure (Doyle et al, 1980; Luckins et al, 1986) but the mechanisms regulating sequential VSG expression are unknown (Borst and Cross, 1982; Bernards, De lange, Michels, Liu, Huisman and Borst, 1984). VSG genes are expressed only when located at chromosome telomeres (Laurent, Pays, Van der Werf, Aerts, Magnus, Van Meirvenne and Steinert, 1986) and M-VATs probably represent expressions of a subclass of pre-existing telomeric VSG genes (Lenardo, Rice-Ficht, Kelly, Esser and Donelson, 1984; Turner, Barry and Vickerman, 1986).

The metacyclic population is antigenically heterogeneous although comprising of only a small proportion of the entire VAT repertoire (Le Ray et al, 1978). In T. congolense, one stock has been shown to contain only 12 M-VATs and in T. b. rhodesiense the repertoire is also limited (Barry et al, 1983; Esser et al, 1982; Turner, Barry, Maudlin and Vickerman, 1988). The M-VATs of a given stock are predictable in both their prevalence and presence in the metacyclic population although the predictability of some M-VATs does not persist over repeated tsetse fly transmission (Barry et al, 1983). The M-VATs continue to be expressed and re-expressed following transmission of metacyclics to their mammalian hosts and their transformation to bloodstream forms (Barry, Hajduk, Vickerman and Le Ray, 1979; Esser and Schoenbechler, 1985; Barry et al, 1983). There is also evidence that M-VATs are expressed in infections initiated from bloodstream forms of T. b. rhodesiense (Barry et al, 1983), T. congolense (Nantulya, Musoke, Rurangirwa and Moloo, 1984) and T. vivax (Nantulya, Musoke and Moloo, 1986), therefore indicating that the expression of M-VATs is not restricted to metacyclic trypanosomes.

2.5 PATHOGENESIS OF TRYPANOSOMA CONGOLENSE

Trypanosomiasis in laboratory and domesticated animals is characterized by the development of anaemia (Dargie, Murray, Murray, Grimshaw and McIntyre, 1979) and immunosuppression (Goodwin, 1970). Anaemia has been considered as the cardinal sign and major disease promoting factor of bovine trypanosomiasis (Hornby, 1921; Murray, 1979). Immunosuppression has been described as one of the most important pathogenic mechanisms in trypanosomiasis because infected

animals generally die not as a direct result of trypanosome activity but as a result of secondary bacterial and viral infection (Tizard, Mellors and Nielsen, 1980).

2.5.1 Anaemia

The development of anaemia is a characteristic occurrence in cattle infected with T. congolense (Fiennes, 1950; 1954) and the disease has been divided into three stages according to the absence or presence of trypanosomes in the bloodstream and on clinical and pathological findings (Murray, 1979; Dargie et al, 1979). The first stage may last from three to twelve weeks and the onset and severity of anaemia correlates with the level and duration of parasitaemia (Jennings, Murray, Murray and Urquhart, 1974; Holmes and Mammo, 1975; Holmes and Jennings, 1976). The anaemia during this stage is haemolytic and intravascular in origin (Fiennes, 1954; Jennings et al, 1974) although extravascular haemolysis has also been described (Naylor, 1971). During stage one anaemia, splenomegaly is a consistent finding (Murray, 1979) and is thought to be due to a combination of lymphoid hyperplasia and erythrophagocytosis (MacKenzie and Cruickshank, 1973; Jennings et al, 1974; Murray et al, 1974). In many animals death occurs at this stage of infection. However, in some animals a further two stages of anaemia are observed.

Stage two of the anaemia is characterized by intermittent low level anaemia. The haemolysis observed in stage one is continued and associated with sustained hyperactivity of the mononuclear phagocyte system (Murray, 1979). Despite this, splenomegaly is no longer prominent. Stage three is characterized by the absence of

trypanosomes in the blood and the animals may either remain anaemic or recover. Death may still occur during stages two or three of anaemia (Murray, 1979).

2.5.2 Immunosuppression

Although in many inbred mouse strains animals become immunosuppressed soon after experimental infection with African trypanosomes,in most domesticated ruminants the most severe immunological changes occur as a result of chronic parasitaemia (Shapiro and Pearson, 1986). Late in infection the lymphoid organs become depleted of lymphoid cells and a systemic neutropenia develops. Lymph nodes shrink and patchy fibrosis replaces the earlier proliferating lymphocytosis (Fiennes, 1970). Although the level of circulating antibody remains high (Luckins and Mehlitz, 1976) the ability to mount specific antibody responses to new antigens decreases (Ackerman and Seed, 1976; Hudson and Terry, 1979).

The mechanisms involved in immunosuppression have been the subject of much debate. In mice, immunological and pathological changes are most apparent in the spleens of infected animals (Kar, Roelants, Mayor-Withey and Pearson, 1981; Wellhousen and Mansfield, 1980) and result in a state of immunosuppression in which both humoral and cellular responses are affected (Corsini, Clayton, Askonas and Ogilvie, 1977; Kar et al, 1981; Wellhousen and Mansfield, 1980; Jaywardena and Waksman, 1977). The reasons for this generalized immunosuppression are unknown; however, there is evidence for the involvement of suppressor cells (Jaywardena and Waksman, 1977), soluble suppressor substances (Albright, Albright and Dusanic, 1977;

Tizard, Nielson, Seed and Hall, 1978) and differential macrophage activity (Grossinsky and Askonas, 1981). It has also been proposed that macrophage dysfunction may play a role in immunosuppression due to trypanosomiasis (Bagasra, Schell and Le Frock, 1981). There is a relative depletion of macrophages bearing Ia antigens in the lymph nodes and spleens of chronically infected animals. These cells are essential in antigen presentation and Bagasra et al (1981) proposed that the depletion of these cells might reduce the host's ability to respond to new antigens. It is probably true that most if not all of these changes influence immunosuppression of the host depending on the stage of infection during which the experiments are performed.

Any hypothesis proposed to explain hyporesponsiveness must consider the following observations. Firstly, IgM effective against new trypanosomal surface antigens continues to be produced throughout the course of a chronic infection (Hudson and Terry, 1979) and total serum IgM levels remain high throughout chronic infections (Luckins and Mehlitz, 1976; Whittle, Greenwood, Bidwell, Bartlett and Voller, 1977). These observations could be explained if the antibody production is T-lymphocyte-independent (Clayton, Ogilvie and Askonas, 1979; Hudson and Terry, 1979) because it appears that immunosuppression does not extend to some T-cell-independent antigens (Mansfield and Bagasra, 1978). Secondly, immune functions return rapidly after chemotherapy of trypanosome infected mice (Roelants, Pearson, Morrison, Mayor-Withey and Lundin, 1979). Presumably, immunoregulatory events occur rapidly enough so that within a few days immunosuppression in these animals is reversed.

Living trypanosomes may release a factor that blocks the terminal stages of differentiation of parasite activated B-lymphocytes to immunoglobulin secreting cells, or inhibits secretion of immunoglobulin by those cells (Black, Sendashonga, O'Brien, Borowy, Naessens, Webster and Murray, 1985). This could explain the rapid production of specific antibody after drug cure but it does not explain the continued IgM production during chronic infection.

The degree of derangement of the immune system during trypanosome infections in cattle is much less marked than in laboratory animals. Therefore, cattle continue to produce antibodies to the trypanosome for many months after infection (Nantulya, Musoke, Barbet and Roelants, 1979), they show less immunosuppression to unrelated antigens and although the lymphoid organs show marked hyperplasia there is less disruption of the tissue architecture than in laboratory animals (Morrison and Murray, 1979; Musoke and Morrison, 1981).

Attempts to evaluate the immune competence of trypanosome infected cattle have concentrated mainly on examining antibody responses to various viral and bacterial antigens. Slight depressions were observed in host antibody responses to Clostridium oedematiens and foot-and-mouth disease (FMD) virus vaccines in naturally infected cattle and cattle experimentally infected with T. congolense (Holmes, Mammo, Thomson, Knight, Lucken, Murray, Jennings and Urquhart, 1974; Scott, Pegram, Holmes, Pay, Knight, Jennings and Urquhart, 1979). Also, cattle infected with T. congolense showed a delay of four days in the antibody response to the hapten dinitrophenyl although the peak titres of antibody were the same as in control cattle; in the same study, infected cattle were found to have

normal responses to parainfluenza virus and to an Escherichia coli bacterophage (Sollod and Frank, 1979). Significant suppression of the antibody response to louping ill virus was reported in cattle experimentally infected with T. congolense (Whitelaw, Scott, Reid, Holmes, Jennings and Urquhart, 1977) and profound suppression of the antibody response to Brucella abortus (S-19) was observed in T. congolense infected cattle (Rurangirwa, Musoke, Nantulya and Tabel, 1983).

2.6 STRATEGIES FOR THE CONTROL OF TRYPANOSOMIASIS

In the absence of suitable immunological control measures for bovine trypanosomiasis, practical control measures include the eradication and control of Glossina populations, the exploitation of trypanotolerant cattle and the administration of antitrypanosomal drugs to cattle (Morrison, Murray and McIntyre, 1981).

2.6.1 Tsetse control

There are two broad categories for the control of tsetse flies, chemical and non-chemical. Chemical control consists of the application of insecticides either from the ground by knapsack spraying or from the air either by helicopter or fixed wing aircraft. Three compounds have been used successfully in large scale field programmes: dichlorodiphenyltribroethane (DDT), dieldrin and endosulfan.

DDT was the first synthetic insecticide to be widely used. Due to its residual effect it is usually used on a single administration basis since deposits can remain lethal for flies for up to one year

after application. Dieldrin, like DDT is a residual insecticide and trials have shown it to be more effective than DDT due to a greater persistence of lethal deposits, particularly in areas of high rainfall (Burnett, Robinson and Le Roux, 1957). Endosulfan is classed as a non-residual insecticide and is therefore less persistent than DDT and Dieldrin. Its solubility in spray solvents makes it suitable for administration by aircraft (Hocking, Lee, Beesley and Matechi, 1966).

The use of insecticides in tsetse control programmes can produce environmental pollution and unwanted ecological effects (Koeman, Rijksen, Smies, Na'Isa and MacLennan, 1971). Of the three insecticides mentioned, repeated aerial application of endosulfan appears to have less general ecological impact (Gray, 1983). Another disadvantage of chemical control is that the application of insecticides is not suited to all ecological zones. Areas of high rainfall and dense vegetation tend to limit the feasibility and efficacy of insecticidal application (Jordan, 1974).

There have been a variety of non-chemical control methods used to reduce Glossina populations in certain areas. The clearance of vegetation and encouragement of human settlement and agricultural development to create an adverse environment for tsetse are long practised and successful techniques (Jordan, 1974). In addition, the destruction of game animals to decrease the flies' food source and therefore the size of the natural infection reservoir has been widely practised (Cockbill, 1967).

A wide variety of tsetse traps have been designed and used for ecological studies and in recent years traps have been re-examined to determine if their catching efficiency could be improved to enable

their use in controlling Glossina populations. It has been demonstrated that the efficiency of traps could be greatly improved if a source of natural host odour (Vale, 1974; Hargrove and Vale, 1979) or a simulated host odour of carbon dioxide and acetone (Vale, 1980) was associated with the trap. A chemical compound, 1-Octen-3-ol has been identified as a potent olfactory stimulant for some species of Glossina and in the field has been shown to be both attractive on its own and with carbon dioxide and acetone (Bursell, 1984; Hall, Beevor Cork, Nesbitt and Vale, 1984). These developments involving tsetse trapping might possibly play a role in future tsetse control programmes.

A sterile insect technique (SIT) has also been investigated. This technique entails rearing large numbers of male flies, sterilising them and then releasing them into the natural habitat. They compete with wild males for mates and reduce the reproductive potential of the females. A small scale trial was conducted with G. m. morsitans on an island on Lake Kariba, Zimbabwe. After the release of puparia which had been dipped in five per cent tepa, 98% control of tsetse was obtained in nine months (Dame and Schmidt, 1970). Failure to achieve G. m. morsitans eradication with the same technique in other areas has been attributed to inadequate isolation barriers and the resultant immigration of flies (Williamson, Dame, Gates, Cobb, Bakuli and Warner, 1983).

2.6.2 Trypanotolerance

Trypanotolerance was first described at the beginning of the twentieth century when certain indigenous taurine cattle breeds

of West Africa and in particular the N'Dama and West African shorthorn were observed to survive and be productive in tsetse infested areas (Pierre, 1906). These two breeds survive in large numbers without the aid of trypanocides in areas where significant tsetse risk exists (ILCA, 1979). In addition to these two breeds of cattle, many species of wild Bovidae have also been shown to exhibit innate resistance to trypanosomiasis (Murray, Morrison and Whitelaw, 1982).

N'Dama and West African shorthorn breeds constitute approximately five per cent of the total cattle population in the 38 countries where Glossina occur (ILCA, 1979; FAO/WHO/OIE, 1982). It is estimated that without any additional control measures, an area of about two million square kilometres in West and Central Africa is suitable for raising trypanotolerant cattle (FAO, 1976). Their failure to be exploited has been because of the breeds' small size and assumed low productivity. However, in areas of low or nil tsetse challenge their productivity has been shown to be much higher than was originally believed (ILCA, 1979).

Trypanotolerance is thought to have developed through natural selection by constant exposure to infection over many generations. However, although resistance to infection probably has a genetic basis, it is not absolute since productivity appears to decrease as the level of challenge increases. Thus N'Dama may exhibit stunting, wasting and abortion associated with trypanosomiasis (ILCA, 1979). Murray et al (1982) have shown that trypanotolerance is innate and is not solely associated with acquired resistance to local trypanosome populations. Using animals with no previous exposure to trypanosomiasis, N'Dama were significantly more resistant to trypanosomiasis

than Zebu and imported exotic breeds in terms of productivity and survival. The prevalence, level and duration of parasitaemia and the severity of anaemia were significantly less in the trypanotolerant cattle. Trypanotolerance has also been attributed to differences in immune responsiveness because of the control of parasitaemia in infected animals. (Akol, Authie, Pinder, Moloo, Roelants and Murray, 1986).

Some individuals of trypanotolerant breeds have been reported as being more susceptible to infection than others (Roelants, 1986). However, other reports have suggested that N'Dama in particular, as well as being trypanotolerant are more resistant to tick-borne diseases such as heartwater, anaplasmosis and babesiosis (Epstein, 1971) and streptothricosis (Coleman, 1967).

2.6.3 Chemotherapy

Chemotherapy of animal trypanosomiasis relies mainly on the use of four compounds: homidium, quinapyramine, diminazene and isometamidium salts; suramin can be added to this list because it is still used to treat trypanosomiasis caused by T. evansi (Jordan, 1986). A summary of the current situation in the chemotherapy of domesticated animals is shown in Table 2.3.

The development of chemotherapy has been accompanied by the development of drug resistance (Williamson, 1979). Drug resistance appears to be acquired by exposure to trypanosomes at sub-therapeutic levels, thereby facilitating selection of a drug resistant sub-population (Whiteside, 1962; Leach and Roberts, 1981). Sub-therapeutic drug levels may occur because of incorrect dosage, a high

TABLE 2.3

Chemotherapy of trypanosomiasis in domesticated animals.

(From Peregrine, 1987)

Generic name	Compound class	Trade Name	Type	Dose and route (mg/kg)	Susceptible trypanosomes	Animal	Comments
Suramin	Sulphonated naphthylamine	Naganol[a]	C	7.0–10.0 i.v.	*T. evansi, T. brucei, T. equiperdum*	Camels	Suramin resistant infections can be treated with quinapyramine
Quinapyramine dimethylsulphate	Quinoline pyramidine	Trypacide sulphate[b] Noroquine[c]	C	3.0–5.0 s.c. 3.0 s.c. 3.0–5.0 s.c.	*T. evansi,* *T. equinum, T. equiperdum* *T. brucei (T. congolense, T. vivax)*	Camels Horses Cattle, small ruminants, pigs, dogs	Active against suramin-resistant strains. Dose should be administered in halves separated by a 6 hour interval. Infection in cattle involving *T. vivax* use 5mg kg^{-1}
Quinapyramine dimethylsulphate: chloride 3:2 w/w	Quinoline pyrimidine	Trypacide prosalt[b] Noroquin prosalt[c]	P	As sulphate	As sulphate	Horses, camels	As sulphate
Diminazene aceturate	Aromatic diamidine	Berenil[d]	C	3.5–7.0 i.m.	*T. vivax, T. congolense (T. brucei, T. evansi)*	Cattle, small ruminants, dogs	Active against homidium resistant infections. Inactive against *T. simiae*
Homidium bromide Homidium chloride	Phenanthridine	Ethidium[e] Novidium[b]	C	1.0 i.m.	*T. vivax, T. congolense*	Cattle, small ruminants, horses, pigs	Bromide soluble only in hot water. Chloride soluble in cold water. Either salt can be used as a sanative pair with diminazene aceturate
Isometamidium chloride	Phenanthridine aromatic amidine	Samorin[b] Trypamidium[f]	C P	0.25–0.5 i.m. 0.5–1.0 i.m.	*T. vivax, T. congolense (T. brucei)*	Cattle	Soluble in water and is heat sensitive. Can be used for *T. brucei* in dogs – use 0.5–1.0 mg kg^{-1}

a Bayer Co., Leverkusen, W. Germany
b May & Baker Ltd., Dagenham, England
c Norbrook Lab. Ltd., Newry, N. Ireland
d Farbwerke Hoechst AG., Frankfurt, W. Germany
e FBC Ltd., Cambridge, England
f Rhone Merieux, Lyon, France

C = Curative
P = Prophylactic
i.m. = intramuscular
i.v. = intravenous
s.c. = subcutaneous
( ) = less susceptible

incidence of trypanosomiasis, irregular treatment with prophylactics or stopping the usage of a prophylactic drug whilst animals are still at risk (Davey, 1950, 1957; Whiteside, 1960).

Resistance to quinapyramine and homidium has developed rapidly and in many countries the only widely used trypanocides are isometamidium and diminazene. Whereas isometamidium has both curative and prophylactic properties, diminazene is rapidly eliminated from treated animals and should be considered only as a curative drug although some prophylactic activity has been reported in cattle for up to three weeks (Van Hoeve and Cunningham, 1964). There are three main reasons for the success of diminazene; it has a much higher therapeutic index in cattle than any other active curative or prophylactic drug; it is active against cattle infections resistant to other drugs and it has not given rise to any serious degree of drug resistance despite widespread use (Williamson, 1970).

Chemoprophylaxis requires stricter supervision of drug administration and conditions of animal husbandry than does chemotherapy. To be an effective prophylactic a drug must be slowly eliminated from a treated animal. Eventually, the drug concentration in the tissues will drop to a level insufficient to kill trypanosomes and under such conditions drug resistance may develop. It is therefore important to administer a second dose of drug before the level of drug has dropped to the critical level below which trypanosomes can survive and multiply. Such a regime requires the continuing presence of trained staff, reliable transport and access to the animals involved (Leach and Roberts, 1981). Chemoprophylaxis is therefore inappropriate for use with nomadic or semi-nomadic livestock in pastoral management

systems. However, using isometamidium the system has been successfully employed on livestock maintained on ranches (Trail, Sones, Jibbo, Durkin, Light and Murray, 1985).

No new trypanocide for domesticated livestock has been marketed for over 25 years primarily because of the prohibitive costs in development of a new drug (Hutner, 1977; Goodwin, 1978) and also the relatively small trypanocide market (Leach and Roberts, 1981). In addition to the lack of new trypanocides, the effectiveness of the current drugs has been threatened because of the development of drug resistance. If further deterioration of the situation is to be prevented, greater expenditure on testing and surveillance for drug resistance is required. However, because of logistical and financial problems such as drug costs, lack of funds to implement control programmes, scarcities of diagnostic services and the lack of well trained permanent staff, extreme limitations are imposed on the application of trypanocides in the field (Holmes and Scott, 1982; Murray and Gray, 1984). In addition, there is an increasing gap between the need for treatment and actual administration of chemotherapy (MacLennan, 1980).

CHAPTER THREE

TRYPANOSOME *IN VITRO* CULTURE

3.1 INTRODUCTION

3.1.1 Background

The main aim of culturing trypanosomes *in vitro* is to enable specific life cycle stages to be produced in large numbers in a controlled environment free from the complicated physiological and immunological systems of their insect vectors and mammalian hosts (Gray *et al*, 1987). It has been possible to culture trypanosomes *in vitro* since *T. lewisi* was successfully maintained in the water of synersis on a nutrient agar slope supplemented with defibrinated rabbit blood (Novy and MacNeal, 1903). African pathogenic trypanosomes proved more difficult to cultivate and for 75 years only one stage in the parasites' life cycle was grown successfully *in vitro* - the uninfective procyclic form. During this time such cultures of *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense* and *T. vivax* were reported to have acquired infectivity (Trager, 1959; Cunningham, 1977; Cunningham and Honigberg, 1977) but continuous infectivity from *in vitro* culture could not be achieved. More recently, the increase in defined or semi-defined media and the improvement in general culture techniques has led to progress in trypanosome culture and now all stages in the life cycles of *T. brucei*, *T. congolense*, *T. vivax* and *T. evansi* can be grown *in vitro*.

Maintenance of tsetse fly colonies can be difficult and many laboratories are unable to handle tsetse flies because of either technical or legal restrictions. Cultured insect form trypanosomes have provided material for serological studies including serodiagnosis (Crowe et al, 1983; Nantulya, Musoke, Rurangirwa, Minja and Saigar, 1985; Luckins et al, 1986; Richardson, Jenni, Beecroft and Pearson, 1986; Pearson et al, 1986; Katende, Nantulya and Musoke, 1987), biochemistry of insect forms (Kilgour, 1980; Betschart, Wyler and Jenni, 1983; Opperdoes and Steiger, 1981; Hart, Misset, Edwards and Opperdoes, 1984; Ross, Cardoso de Almeida and Turner, 1987) and molecular biological studies (Overath, Czichos, Stock and Nonnengaresser, 1983).

Bloodstream form culture systems offer an alternative to laboratory animals particularly in cases involving trypanosome stocks or species that are difficult to establish in vivo. However, trypanosome yields from bloodstream form cultures remain modest in comparison with those attained from infections of laboratory animals. Trypanosomes derived from bloodstream form cultures have been used to examine antigenic variation free from host influences (Doyle et al, 1980; Luckins, et al, 1986; Prain and Ross, 1988), species identification by way of the blood incubation infectivity test (Jenni and Brun, 1982) and for drug testing (Balber, Gonias and Pizzo, 1985; Borowy, Fink and Hirumi, 1985a,b,; Borowy, Hirumi, Waithaka and Mkoji, 1985c; Borowy, Nelson, Hirumi, Brun, Waithaka, Schwarz and Polak, 1988).

3.2 BLOODSTREAM FORM CULTURES

3.2.1 T. brucei

The co-cultivation of trypanosome bloodstream forms and a mammalian cell feeder layer was used to successfully maintain T. brucei at 37°C for three days using mouse L-cells and NCTC-109 medium (Le Page, 1967). This idea was developed further ten years later when bloodstream trypomastigotes were cultivated in the presence of bovine fibroblast type cells, buffalo lung or Chinese hamster lung tissue culture cells with HEPES buffered RPMI 1640 supplemented with 20% FBS (Hirumi, Doyle and Hirumi, 1977; Hill, Shimer, Caughey and Sauer, 1978a). The majority of the organisms grew in the culture supernatant but often entered the intercellular spaces in cultures older than eight days. Although trypanosomes grown in this system retained their infectivity for mammalian hosts and displayed variant antigen on their surface, they were monomorphic and morphologically indistinguishable from long slender bloodstream forms. As such they were not transmissible to tsetse flies. Pleomorphic forms of T. brucei were cultivated by Hill et al (1978b) but could only be maintained in the short term. Long term cultivation of vertebrate infective bloodstream-like forms of pleomorphic stocks of T. b. brucei and T. b. rhodesiense transmissible by tsetse fly was eventually achieved using Eagle's Minimal Essential Medium (EMEM) supplemented with 15-20% non-commercially prepared serum from various sources; for example, rabbit, horse, goat, sheep and human, in the presence of a mammalian cell feeder layer (Brun, Jenni, Tanner,

Schonenberger and Schell, 1979; Brun, Jenni, Schonenberger and Schell, 1981; Brun, Hecker, Jenni and Moloo, 1984).

3.2.2 T. evansi

A number of different isolates of T. evansi bloodstream trypomastigotes have also been successfully cultured. One stock isolated from Java was maintained in RPMI 1640 medium supplemented with 20% heat inactivated rabbit serum with a feeder layer of rabbit fibroblasts at 37°C (Zweygarth, Ahmed and Rehbein, 1983). Three stocks of African origin were cultured with swine fibroblastoids with RPMI 1640, 20% heat inactivated horse serum with 10% Leibovitz medium also at 37°C (Zweygarth and Rottcher, 1986).

3.2.3. T. congolense

Primary cultures of an uncloned stock of T. congolense were established from trypanosomes present in bovine dermal explants (Gray, Brown, Luckins and Gray, 1979). The cultures were initiated from local skin reactions at the sites of bites by G. m. morsitans and incubated at 37°C. They showed parasite multiplication and retention of infectivity for up to 21 days. However, these cultures could not be sub-passaged and this was attributed to extensive cellular outgrowth from the explants and to possible immunological factors included with explants from the calves (Gray et al, 1979).

The method of Gray et al (1979) was improved on by replacing the dermal explant with a feeder layer of bovine aorta endothelial cells (Gray, Ross, Taylor, Tetley and Luckins, 1985; Ross, Gray, Taylor and Luckins, 1985). While infective forms grown in this system could be maintained at 28°C or 37°C in FBS or NBS, at 28°C in FBS trypanosomes

were capable of differentiating into procyclic forms (Gray *et al*, 1985). The trypanosomes also induced local skin reactions in rabbits, unlike trypomastigotes derived from the bloodstream of infected hosts and were therefore designated mammalian forms, possibly resembling parasites which develop extravascularly in the vertebrate host following the introduction of metacyclics into the skin by infected tsetse fly bite. This could be related to the routine maintenance of the trypanosomes at 28°C rather than 37°C and because these cultures were established using *in vitro* derived metacyclic trypanosomes. A successful but inconvenient method involving adult and foetal goat serum supplemented RPMI was used to cultivate one stock of *T. congolense* for 93 days (Hirumi and Hirumi, 1984).

T. congolense bloodstream forms form a close association with the endothelial cell feeder layer where the majority of trypanosomes attach. In contrast, *T. brucei* group trypanosomes invade the intercellular spaces of the fibroblast feeder layer and also multiply in the culture supernatant. This probably relates to the trypanosome's behaviour in the mammalian host where *T. congolense* is mainly a parasite of the vasculature and *T. brucei* group trypanosomes invade the extravascular sites such as the lymphatics and tissue spaces as well as the bloodstream.

3.2.4 *T. vivax*

A culture system has been developed for a rodent adapted West African stock of *T. vivax* (Brun and Moloo, 1982). Animal infective trypomastigotes were successfully grown in suspension culture over fibroblast-like cells for three months using MEM with Earle's salts, supplemented with 20% heat inactivated goat serum.

3.3 CELL FREE SYSTEMS

Abolishing the requirement for mammalian cell feeder layers has been a recent achievement of trypanosome culture research (Brun and Jenni, 1987). Bloodstream forms of the T. brucei subgroup, T. evansi and T. equiperdum can now be grown axenically by the addition to the medium of sulphydryl reducing agents, which are normally excreted by feeder layers. In one system, 2-mercaptoethanol or thioglycerol were used to provide the reducing conditions (Baltz, Baltz, Giroud and Crockett, 1985) whilst cysteine was used in another system (Duszenko, Ferguson, Lamont, Rifkin and Cross, 1985). Both of these systems require an adaptation period on a mammalian cell feeder layer.

As with all in vitro cultures the number of stocks able to be maintained in those cell free systems is limited. It remains to be seen how easily other stocks become adapted and whether the culture forms have the same characteristics as those derived from the mammalian host or those maintained with feeder layers.

3.4 PROCYCLIC CULTURES

Procyclic forms of T. brucei differentiate from bloodstream forms in culture at a similar rate to that observed in the insect host and show similar morphology to their vector counterparts (Brown, Evans and Vickerman, 1973). They can be grown in large quantities in complex, semi-defined or defined media (Trager, 1978; Brun and Schoenberger, 1979; Cunningham, 1977; Cross and Manning, 1973). The

frequency of passage can affect trypanosome differentiation *in vitro*. If procyclics are kept in the logarithmic phase of growth by frequent dilution with growth medium they remain at this stage of the life cycle. However, if *T. congolense* procyclics are allowed to enter stationary phase the population will differentiate into non-dividing proventricular forms (Reichenow, 1934 cited by Gray *et al*, 1987). Similar findings have been reported for *T. brucei* (Evans and Brown, 1972; Trager, 1959; Cunningham and Honigberg, 1977). Procyclic and proventricular forms appear to be insensitive to antibiotics although the presence of antibiotics in cultures of these trypanosomes prevents their differentiation to epimastigotes.

3.5 EPIMASTIGOTE CULTURES

In the absence of antibiotics and in the presence of a suitable substratum the procyclic/proventricular forms attach to that substratum and differentiate to the epimastigote stage (Gray *et al*, 1987). The attachment is via the flagellar tip and is a feature of kinetoplastid flagellates (Hommel and Robertson, 1976). Epimastigote attachment is necessary for the production of metacyclic trypanosomes (Hendry and Vickerman, 1988), although *in vitro* requirements vary from species to species as a reflection of differences in the life cycle *in vivo*.

3.5.1 *T. brucei*

In the tsetse fly, the epimastigotes of *T. brucei* differentiate into metacyclic forms while attached to the microvilli of the salivary gland. This close association *in vivo* between *T. brucei* epimastigotes and tsetse salivary gland epithelium is reflected in

the *in vitro* culture requirements of *T. brucei*. Successful *in vitro* culture of infective metacyclic forms of *T. b. brucei* and *T. b. rhodesiense* at 27°C using Cunningham's medium in the presence of head and salivary gland or abdominal explants of *G. m. morsitans* or *Phormia regina* has been reported recently (Kaminsky, Beaudoin and Cunningham, 1987, 1988).*

3.5.2 *T. vivax*

The developmental stages of *T. vivax* in the tsetse fly occur only within the proboscis. The production of insect form *in vitro* cultures consisting of elongated trypomastigotes, epimastigotes and metacyclic forms requires a bovine fibroblast feeder layer and 0.5% Matrix Gel Green A beads (Amicon) at 25°C (Hirumi, Nelson and Hirumi, 1983; Hirumi, Hirumi, Nelson and Moloo, 1984; Hirumi, Nelson, Hirumi and Moloo, 1985). It is unclear why such a system containing feeder cells is necessary to cultivate these stages of *T. vivax*.

3.5.3 *T. congolense*

The first cultures containing epimastigote forms of *T. congolense* were described by Steiger, Steiger, Trager and Schneider (1977) where trypanosomes were maintained along with a continuous cell line of *G. morsitans*. The epimastigotes did not attach, however, and the cultures never became infective to mammalian hosts.

The principles employed in attempts to establish bloodstream forms of *T. congolense* by Gray *et al* (1979) were adapted and modified in attempting to culture the insect forms of the parasite. Cultures were initiated from the mouthparts of infective *G. m. morsitans* using dermal explants from immunologically naive hosts and incubating the cultures at 28°C in EMEM supplemented with Earle's salts, FBS and

* (Cunningham, 1986; Cunningham and Kaminsky, 1986)

L-glutamine (Gray et al, 1981). The trypanosomes multiplied rapidly and eventually formed a layer of mainly adherent epimastigotes on the inert surface of the culture flask. The cultures became infective to mice from day 14 post-initiation, presumably when metayclic forms were first produced. Primary cultures could be passaged in supplemented medium without dermal explants while retaining their infectivity. All cultures included a variety of morphologically different forms closely resembling those from Glossina. Another method of obtaining metacyclic forms in vitro whereby the bovine dermal collagen explant was replaced by commercially available purified dermal collagen (Vitrogen) used trypanosomes from infected blood rather than infected tsetse fly mouthparts (Hirumi, Hirumi and Moloo, 1982).

The successful establishment in vitro of two cloned stocks of T. congolense, one from East Africa and one from West Africa, again used different developmental stages from the fly (Gray et al, 1984). Cultures were established using different support media and their growth characteristics in primary and sub-passaged cultures examined. MEM was considered to be better than RPMI 1640 or M199 and became the medium of choice. However, MEM older than four months consistently produced fewer numbers of metacyclic forms. The foetal bovine serum, another important component of the system was batch tested as different serum preparations affected growth and differentiation resulting in considerable variation in the numbers of metacyclics produced in vitro.

Few stocks of T. congolense are cultured in vitro and therefore relatively little is known about the characteristics of different

stocks isolated from different areas. The vast increase in surface area from a tsetse fly proboscis to a T-25 tissue culture flask gives this culture system the ability to produce large numbers of metacyclic forms for use in immunological and biochemical studies.

The work undertaken in this Chapter describes the adaptation to *in vitro* culture of six Zambian stocks of *T. congolense*. The characteristics of each stock *in vitro*, the production of metacyclic forms, their infectivity to mice and their ability to produce local skin reactions in rabbits are presented and discussed in relation to previous work on *in vitro*-derived insect forms of *T. congolense*.

3.6 MATERIALS AND METHODS

3.6.1 Animals

Female outbred albino mice (TO) (Tuck and Son) were used in cloning trypanosomes, preparation of bloodstream form stabilates, infectivity titrations, and infecting tsetse flies. The mice were kept in plastic cages with metal tops through which food (rat cake - Oxoid Laboratory Animal Diets) and water were added *ad libitum*. Up to six mice were kept in one cage on wood shavings and water absorbent granules (Oakite supplied by Oakite Ltd., Retford, Nottingham). Each animal was identified by individual markings with picric acid.

New Zealand White (NZW) female rabbits (Bantin and Kingman) were used for maintaining tsetse flies. The rabbits weighed 2-3 kg and each was kept in an individual cage with food (rabbit pellets SG-1, Oxoid Laboratory Animal Diets) and water added *ad libitum*. The

animals were identified with numbered ear tags.

3.6.2 Tsetse flies

Glossina morsitans morsitans puparia were supplied by courtesy of Dr. A.M. Jordan, Tsetse Research Laboratories, Langford, Bristol. The puparia were incubated at 25°C, 70% relative humidity until the tsetse flies emerged. After emergence, the teneral flies were transferred to Geigy-15 cages. Handling of tsetse flies during transfer was facilitated by chilling the flies for four minutes at -20°C.

3.6.3 Trypanosomes

The six stocks of T. congolense used to produce insect form in vitro cultures were obtained as uncloned stabilates from Dr. D.G. Godfrey, London School of Hygiene and Tropical Medicine. The trypanosomes were isolated from dogs and the initial isolates inoculated into Wistar rats. The original isolation numbers and the TREU (Trypanosomiasis Research Edinburgh University) numbers of the uncloned parent stocks and their cloned derivatives are shown in Table 3.1.

For each of the six stocks, the trypanosomes were cloned by diluting infected mouse blood in PSG pH 8.0. A straightened paper clip was used to apply a small drop of this diluted blood to a glass coverslip to make a hanging drop preparation over a well containing guinea pig serum. When a single trypanosome was seen by at least two observers, a syringe containing 0.1 ml PSG was used to wash the droplet into the well. The contents of the well were then drawn back into the syringe barrel. A mouse was put in a restrainer and a

TABLE 3.1

The code numbers of the Zambian *Trypanosoma congolense* isolates used to produce insect form *in vitro* cultures.

Original isolation code	TREU numbers of original isolates	TREU numbers of the cloned derivatives
DA/ZM/81/TRPZ 105	TREU 1842	TREU 1881
DA/ZM/82/TRPZ 132	TREU 1851	TREU 1885
DA/ZM/81/TRPZ 109	TREU 1843	TREU 1894
DA/ZM/82/TRPZ 133	TREU 1852	TREU 1896
DA/ZM/81/TRPZ 114	TREU 1845	TREU 2034
DA/ZM/81/TRPZ 115	TREU 1846	TREU 2037

syringe of PSG with a 26 G hypodermic needle was inserted in to the tail vein. The syringe was carefully removed leaving the needle in the vein and the syringe containing the single trypanosome was then inserted and its contents inoculated into the mouse. This method was repeated at least 10 times for each of the six stocks. For each stock, the infectivity of clones for mice was low and only one mouse per group developed parasitaemia. Only six clones, namely, TREU 1881, TREU 1885, TREU 1894, TREU 1896, TREU 2034 or TREU 2037 were produced. Stabilates were made of these clones and they were used in the production of *in vitro* cultures. Full pedigree charts for these stocks are given in Appendix I.

Metacyclic trypanosomes from *in vitro* cultures of *T. congolense* TREU 1457 were used as controls in some experiments. TREU 1457 is a cloned derivative of TREU 1290 and was originally isolated from an ox

in Zaria, Nigeria in 1967 as Zaria/67/LUMP/69 (Luckins and Gray, 1983).

3.6.4 Infection and maintenance of tsetse flies

Mice were infected intraperitoneally with the cloned trypanosomes TREU 1881, TREU 1885, TREU 1894, TREU 1896, TREU 2034 or TREU 2037 from stabilates. Teneral G. m. morsitans were fed within 72 hours of emergence on anaesthetized mice with rising parasitaemias for each of the six stocks. Subsequently, flies were maintained for 28 days by feeding every 48 hours on the shaven back of a NZW rabbit. Previous experience has shown that after 21 days the majority of infected flies have positive labral infections (Luckins, personal communication; Hoare, 1972; Nantulya et al, 1978b).

3.6.5 Media

EMEM with glutamine and without sodium bicarbonate in powder form (Flow Laboratories or Gibco Europe Ltd) was used in all cultures. The stock medium was supplemented with 4 mM glutamine, 20 mM HEPES buffer, 2.2 g/l sodium bicarbonate and 20% FBS which was heat inactivated at 56^{o}C for 30 minutes before use.

3.6.6 Preparation of dermal collagen explants

Dermal collagen explants were prepared from the flanks of three to six month old calves. An area of skin was shaved before the calf was killed. After death, the area selected was washed with soap and water, shaved more closely and then surface sterilized by washing with 70% ethanol. The skin was excised using a sterile scalpel (Swann-Morton No. 22) and then placed epidermal side down in a

sterile petri dish and transferred to a Microflow hood. Excess connective tissue and small blood vessels were removed from the skin and discarded. Collagen was collected in 5-8 mm wide strips and washed in medium containing 200 IU/ml penicillin and 200 µg/ml streptomycin. After washing, the collagen was cut into small pieces, 3-5 mm^2 and 2 mm thick. Pieces of collagen were transferred to a T-25 culture flask containing medium supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. The flask was gassed in 5% CO_2 in air and stored at 4°C for up to eight weeks.

3.6.7 Preparation of culture flasks before trypanosome isolation

Two days before isolation of *T. congolense* from *G. m. morsitans* bovine dermal collagen explants were placed singly in Falcon Primaria T-25 tissue culture flasks. Two ml medium containing 100 IU/ml penicillin and 100 µg/ml streptomycin was added to each of these flasks and they were kept at 28°C in 5% CO_2 in air.

3.6.8 Dissection of tsetse flies

Tsetse flies were chilled over solid CO_2 before their legs and wings were removed. A fly was then placed in a sterile petri dish containing small pools of medium containing 200 IU/ml penicillin and 200 µg/ml streptomycin. Using sterile forceps and a sterile mounted needle, the head was removed. The mouthparts were dissected by pressing down on the head, releasing the proboscis from the labial palps and allowing the proboscis and attached bulb to be removed with the sterile fine forceps. The proboscis was placed in a pool of medium and examined for trypanosome infection using an inverted microscope. The proventriculus was prepared by gently pressing the

sides of the thorax with sterile ribbed forceps. Using fine forceps, the proventriculus could then be removed as it emerged from the thorax. The midgut was exposed by opening the abdomen. The proventriculus and the midgut were then placed in separate pools of medium and examined microscopically for trypanosome infection.

3.6.9 Establishment of primary insect form culture

Medium was removed from flasks containing a bovine dermal collagen explant and an infected proboscis, proventriculus or midgut was placed alongside the explant. Two ml of medium containing antibiotics was added to each flask. The culture flasks were then incubated at 28°C and left undisturbed for six days. After this time, culture supernatants were changed as necessary, using medium which did not contain antibiotics.

3.6.10 Culture maintenance

Initially, established cultures were maintained in 4 ml of medium which was changed at 48 hour intervals. This routine was changed to involve incubation with 4 ml medium for 48 hour intervals during weekdays, followed by a 72 hour incubation with 8 ml of medium. Established cultures were passaged by transferring $1\text{-}4 \times 10^7$ trypanosomes to a new T-25 culture flask. The most suitable cultures for passaging contained supernatant bundles of epimastigote forms. Flasks containing the passaged trypanosomes were gently shaken to allow the supernatant to cover the entire surface area of the flask bottom. Fresh medium was then added and the flasks were gassed in 5% CO_2 in air and incubated at 28°C.

3.6.11 Conditioned medium

Conditioned medium (CM) was used as an aid to culturing particularly when resuscitating cryopreserved trypanosomes. The CM was prepared from supernatants of established cultures which were centrifuged at 200 *g* for 15 minutes before being filtered through a 0.22 µm filter. Freshly prepared CM was either used undiluted or mixed with fresh medium.

3.6.12 Cryopreservation and re-establishment of culture-derived trypanosomes

Trypanosomes were removed from the flask surface by scraping and the supernatant collected was then mixed with an equal volume of fresh medium containing 15% (v/v) glycerol. This suspension was dispensed into 1.8 ml cryopreservation tubes (Nunc) and cooled to -60°C. The cooling was achieved in one of two ways; either by using a Union Carbide collar at setting F or a Planer R204 Cell Freezer. Setting F on a Union Carbine collar cooled at a rate of -1°C per minute whilst the Planer Cell Freezer was used on a programme which cooled at a rate of -5°C per minute to 4°C then -1°C per minute to -30°C and finally at -2°C per minute until -60°C. After cooling, the tubes were transferred to the liquid nitrogen and stored at -196°C.

For resuscitating cultures, the cell suspension was thawed as quickly as possible by incubating the cryopreservation tube in a water bath at 37°C. The contents were then placed in a T-25 flask and 2 ml of fresh medium was added before gassing the flask in 5% CO_2 in air.

3.6.13 Separation of metacyclic forms from other insect forms

Routinely, metacyclic forms were separated from the other insect forms using diethylaminoethyl (DEAE) cellulose columns (Whatman DE52, Whatman Lab Sales) equilibrated with phosphate buffered saline pH 8.0 containing 1% (w/v) glucose (PSG) (Gray et al, 1984). A volume of packed and equilibrated DE52 equivalent to culture supernate was used for each separation.

When assessing the numbers of metacyclics produced from cultures over a period of time (3.7.4) a more accurate method than that described above was necessary. Two ml of DE52 was packed and equilibrated with PSG in small polystyrene columns (Pierce, UK). A 0.5 ml volume of culture supernatant was added to this and the metacyclic forms eluted with 2.5 ml PSG. Three ml of eluate was collected and the metacyclics counted using an improved Neubauer haemocytometer (Ross, 1987).

3.6.14 Infectivity tests

Primary cultures (3.6.7) were monitored at seven day intervals for the acquisition of infective metacyclic trypanosomes by injecting 0.5 ml of culture supernatant intraperitoneally into each of two mice and monitoring the tail blood for infection for 40 days.

To determine the proportion of in vitro-derived metacyclic forms which were infective to mice, infectivity titrations were carried out as described by Lumsden, Cunningham, Webber, Van Hoeve and Walker (1963).

In each infectivity titration, the number of metacyclic forms was initially adjusted in PSG pH 8.0 to $\log_{10}$ 7.0 trypanosomes per

ml. Serial 10-fold dilutions were then carried out to a final concentration of 10^2 metacyclics per ml. Groups of six mice were then inoculated intraperitoneally with 0.1 ml of each dilution containing 10^5, 10^4, 10^3 or 10^2 trypanosomes and monitored for evidence of infection by wet film examination of tail blood for up to 40 days post-infection.

In addition to the two methods described above, the ability of *in vitro*-derived metacyclic forms to produce local skin reactions in rabbits was examined by inoculating metacyclic trypanosomes intradermally into the shaven backs of NZW rabbits.

3.7 RESULTS

3.7.1 Adaptation to in vitro culture of six Zambian stocks of T. congolense

All six cloned Zambian stocks of *T. congolense* were successfully adapted to insect form *in vitro* culture and the results of those adaptations are presented in Tables 3.2 - 3.7. Each of the different sources of the trypanosomes, proventriculi, proboscides and midguts gave rise to cultures producing infective metacyclic forms although some sources were not successful for all stocks.

Various stages of development of a primary insect form culture taken from smears of culture supernatants of *T. congolense* TREU 1885 are shown in Figures 3.1, 3.2 and 3.3. For the first 10 days in culture the majority of trypanosomes in the culture supernatant showed procyclic morphology, similar to that found in the tsetse fly midgut (Figure 3.1). Fourteen days after attachment was first observed on the flask substratum, small bundles of epimastigotes were

FIGURE 3.1

Procyclic forms of *T. congolense* TREU 1885 from the supernatant of a ten day old culture. These forms are equivalent to those found in the tsetse fly midgut.

(Giemsa stain, x 1,100 magnification)

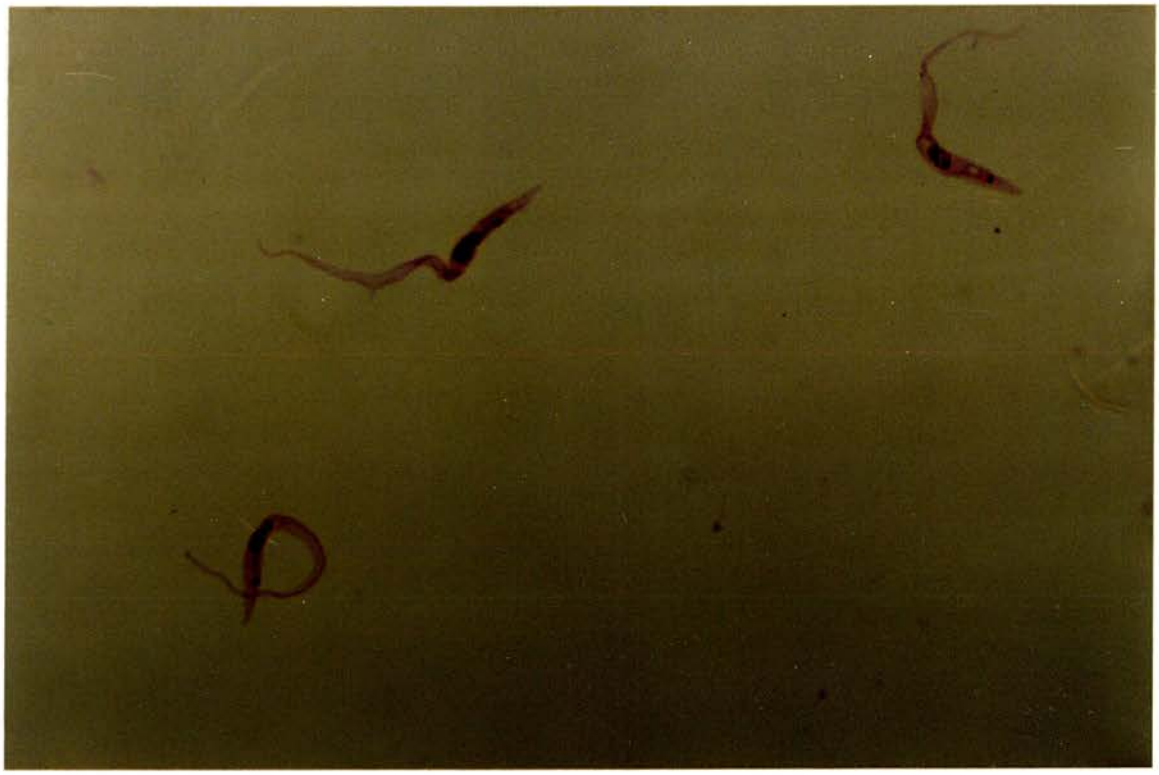


FIGURE 3.2

Epimastigote bundle of *T. congolense* TREU 1885 from the supernatant of a 14 day old culture.

(Giemsa stain, x 1,100 magnification)

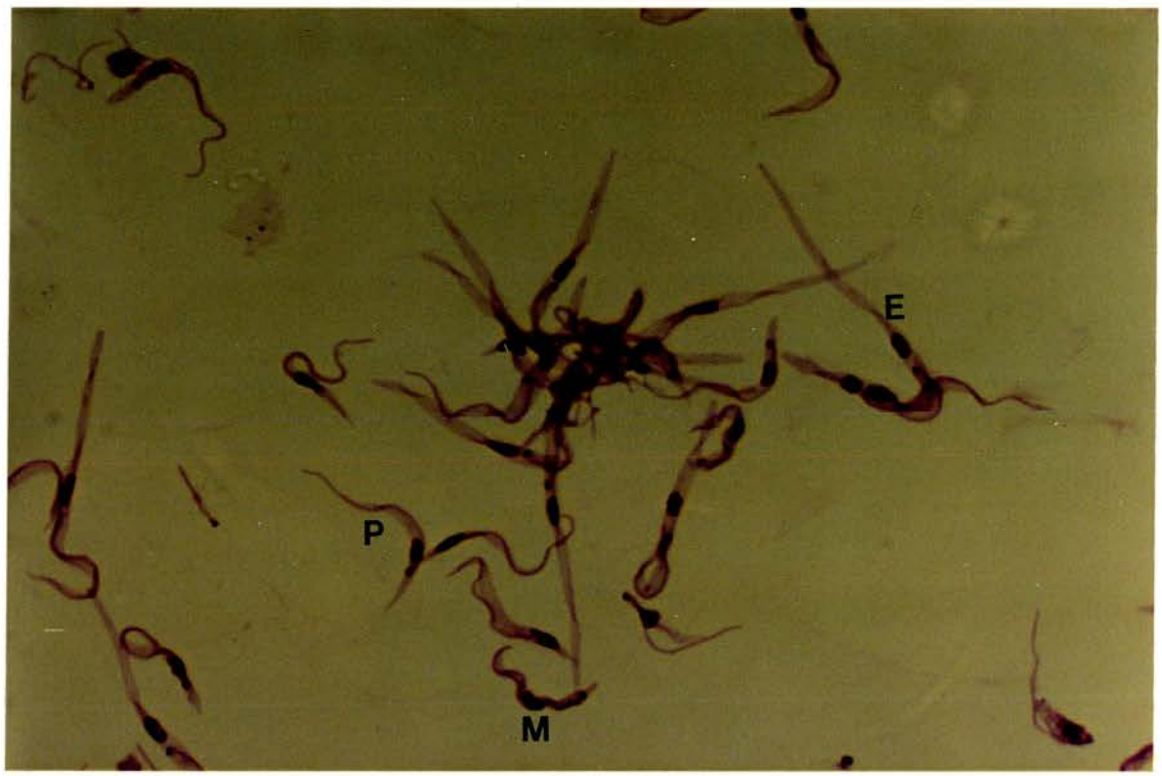
FIGURE 3.3

Insect forms of *T. congolense* TREU 1885 from the supernatant of a mature culture.

Note the length of the epimastigote forms (E) compared to those in Figure 3.2.

Metacyclic (M) and procyclic (P) forms are also present.

(Giemsa stain, x 1,100 magnification)


E
P
M

seen in the supernatant (Figure 3.2) together with the procyclic forms. Finally, in a mature culture all the insect forms were observed in harvested supernatants (Figure 3.3). The greatest proportion of insect forms present were the uncoated procyclic and epimastigote stages with only a small proportion of trypanosomes present being the metacyclic form.

3.7.1.1 TREU 1881

The adaptation of T. congolense TREU 1881 is shown in Table 3.2. Five cultures were initiated from infected proventriculi. All the original parent cultures demonstrated attachment of epimastigote bundles by day 18 post-initiation. Sub-passages were made from four of the original cultures and three of these were successful. However, proventriculus 6, proventriculus 5 parental and passage 1 (p1) were discarded due to death of the culture. The three remaining parental cultures proventriculus 1, 2 and 3 and their sub-passages all became infective to mice by day 25 post-initiation. The parental cultures were cryopreserved on day 41 post-initiation and all TREU 1881 cultures were the derivatives of proventriculus 3.

3.7.1.2 TREU 1885

Two parental cultures initiated with T. congolense TREU 1885 showed epimastigote attachment by day 35 post-initiation (Table 3.3). Of the two successful cultures, proventriculus 3 became contaminated by bacteria and was discarded before it was shown to be infective to mice or cryopreserved. The culture initiated from midgut 5 was successfully sub-passaged three times on days 27, 29 and 33 post-isolation. The parental culture was discarded on day 40 due

TABLE 3.2

Origin, designation, time of attachment, and time of infectivity to mice of *in vitro* cultures of *Trypanosoma congolense* TREU 1881.

Source of trypanosomes	Designation of sub-passaged culture and day (post-isolation) of passage	Attachment of epimastigotes (days)	Infectivity to mice (days)
Proventriculus 1	parental	18	25
	p1; 29	29	+
	p1; 33	33	+
Proventriculus 2	parental	18	25
	p1; 19	19	+
	p1; 23	23	+
	p1; 30	30	+
Proventriculus 3	parental	14	23
	p1; 13	15	+
	p1; 35	35	+
Proventriculus 5	parental	18	-
	p1; 30	-	-
Proventriculus 6	parental	18	-

\+ cultures infective to mice
\- no attachment of epimastigotes and/or no infectivity achieved in mice

to bacterial and fungal contamination. However, the sub-passages became infective to mice by day 44 post-initiation and were successfully cryopreserved on day 56. The insect form cultures designated *T. congolense* TREU 1885 were derivatives of midgut 5 p1.

3.7.1.3 TREU 1894

Cultures of *T. congolense* TREU 1894 were initiated from proventriculi and proboscides of three infected tsetse flies (Table 3.4). Three of the parental cultures, proventriculus 1, proventriculus 2 and proboscis 2 were successful in producing attached epimastigote forms. Although proventriculus 2 and proboscis 2 gave

TABLE 3.3

Origin, designation, time of attachment, and time of infectivity to mice of *in vitro* cultures of *Trypanosoma congolense* TREU 1885.

Source of trypanosomes	Designation of sub-passaged culture and day (post-inoculation) of passage	Attachment of epimastigotes (days)	Infectivity to mice (days)
Proventriculus 1	parental	-	-
Proventriculus 2	parental	-	-
Proventriculus 3	parental	35	-
Proboscis 1	parental	-	-
Proboscis 2	parental	-	-
Midgut 5	parental	21	-
	p1; 27	27	44
	p1; 29	29	+
	p1; 33	33	+
Proboscis 3	parental	-	-
Proboscis 4	parental	-	-

+ cultures infective to mice
- no attachment of epimastigotes and/or no infectivity achieved in mice

rise to attached epimastigotes by days 21 and 23 respectively these cultures had to be discarded due to bacterial contamination before infectivity to mice was obtained. The culture initiated from proventriculus 1 was also discarded due to contamination. However, a second sub-passage (p2) from this culture did eventually give rise to attached epimastigotes by day 89 post-initiation and subsequently became infective to mice on day 104. This culture was cryopreserved and all *T. congolense* TREU 1894 *in vitro* cultures were derived from proventriculus 1 p2.

TABLE 3.4

Origin, designation, time of attachment and time of infectivity to mice of *in vitro* cultures of *Trypanosoma congolense* TREU 1894.

Source of trypanosomes	Designation of sub-passaged culture and day (post-isolation) of passage	Attachment of epimastigotes (days)	Infectivity to mice (days)
Proventriculus 1	parental	33	-
	p1; 37	-	-
	p2; 45	89	104
Proboscis 1	parental	-	-
Proventriculus 2	parental	21	-
Proboscis 2	parental	23	-
Proventriculus 3	parental	-	-
Proboscis 3	parental	-	-

\+ cultures infective to mice
\- no attachment of epimastigotes and/or no infectivity achieved in mice

3.7.1.4 *TREU 1896*

Table 3.5 shows the results of the adaptation *in vitro* of *T. congolense* TREU 1896. Of 11 cultures, four showed attachment of epimastigote forms from between day 9 and day 35 post-initiation. Five cultures were discarded due to fungal and/or bacterial contamination and were shown to be non-infective to mice. However, a sub-passage from the proventriculus 3 parental culture on day 36 post-initiation became infective to mice. These cultures were cryopreserved and subsequent cultures of *T. congolense* TREU 1896 were derivatives of proventriculus 3 p1.

Cryopreservation and resuscitation of TREU 1896 insect form cultures was extremely difficult and cultures of this stock were not

TABLE 3.5

Origin, designation, time of attachment, and time of infectivity to mice of *in vitro* cultures of *Trypanosoma congolense* TREU 1896.

Source of trypanosomes	Designation of sub-passaged culture and day (post-isolation) of passage	Attachment of epimastigotes (days)	Infectivity to mice (days)
Proventriculus 1	parental	-	-
Proventriculus 2	parental	35	-
Proboscis 2	parental	-	-
Proventriculus 3	parental	16	-
	p1; 36	36	110
	p1; 38	38	+
	p1; 49	49	+
Proboscis 3	parental	-	-
Proventriculus 4	parental	-	-
Proventriculus 5	parental	18	-
	p1; 37	37	-
Proventriculus 6	parental	-	-
Proventriculus 7	parental	9	-
Midgut 7	parental	-	-
Midgut 8	parental	-	-

+ cultures infective to mice
- no attachment of epimastigotes and/or no infectivity achieved in mice

Overdulve and Antonisse (1970) have demonstrated that the mathematical presentation of the method of Lumsden *et al* (1963) is incorrect. Therefore, the log ID_{63}/ml values presented in Table 3.8 and Table 3.9 are statistically inaccurate. Nevertheless, the biological interpretation of the data remains valid.

always available for some of the work described here and in subsequent experiments.

3.7.1.5 TREU 2034 and TREU 2037

T. congolense TREU 2034 and TREU 2037 were the last of the Zambian stocks to be isolated in vitro and since proventriculi had been shown to be the most successful source of trypanosomes in producing attached epimastigotes in vitro, only proventriculi from infected flies were used in isolating these stocks. Five cultures of T. congolense TREU 2034 were initiated but only one produced attached epimastigotes (Table 3.6) and although this culture was subsequently discarded due to fungal contamination, a sub-passage from it produced infective metacyclics at day 107 post-initiation. All subsequent insect form cultures of T. congolense TREU 2034 were derived from proventriculus 4 p1.

None of the parental cultures of TREU 2037 produced attached epimastigote forms although two sub-passages from proventriculus 2 were successfully established (Table 3.7). A second sub-passage of proventriculus 2 (p1 day 58) on day 79 post-initiation produced attached epimastigotes five days after passage and became infective to mice at 107 days post-initiation. This culture was successfully cryopreserved and all T. congolense TREU 2037 insect form cultures are derived from proventriculus 2 p2.

3.7.2 Infectivity of in vitro-derived metayclic forms of five Zambian T. congolense stocks

The results of infectivity titrations (Lumsden et al, 1963) of cultures of five of the Zambian T. congolense stocks are shown in Table 3.8. Infectivity values for each of the stocks have to be

TABLE 3.6

Origin, designation, time of attachment and time of infectivity to mice of *in vitro* cultures of *Trypanosoma congolense* TREU 2034.

Source of trypanosomes	Designation of sub-passaged culture and day (post-isolation) of passage	Attachment of epimastigotes (days)	Infectivity to mice (days)
Proventriculus 1	parental	-	-
Proventriculus 2	parental	-	-
Proventriculus 3	p1; 51	-	-
Proventriculus 4	parental	47	-
	p1; 44	44	107
Proventriculus 5	parental	-	-

- no attachment of epimastigotes and/or no infectivity to mice

TABLE 3.7

Origin, designation, time of attachment and time of infectivity to mice of *in vitro* cultures of *Trypanosoma congolense* TREU 2037.

Source of trypanosomes	Designation of sub-passaged culture and day (post-isolation) of passage	Attachment of epimastigotes (days)	Infectivity to mice (days)
Proventriculus 1	parental	-	-
Proventriculus 2	parental	-	-
	p1; 58	62	-
	p1; 63	-	-
	p1; 79	84	107
Proventriculus 3	parental	-	-
Proventriculus 4	parental	-	-
Proventriculus 5	parental	-	-

- no attachment of epimastigotes and/or no infectivity to mice

Overdulve and Antonisse (1970) have demonstrated that the mathematical presentation of the method of Lumsden et al (1963) is incorrect. Therefore, the log ID_{63}/ml values presented in Table 3.8 and Table 3.9 are statistically inaccurate. Nevertheless, the biological interpretation of the data remains valid.

TABLE 3.8

Results of infectivity titrations on cultures of Trypanosoma congolense TREU 1894, TREU 2034, TREU 2037, TREU 1885 and TREU 1881 using an initial concentration of $\log_{10}$ 7.0 metacyclics per ml.

Stock of trypanosomes	Culture	Number of days in vitro	Log_{10} ID_{63}/ml
TREU 1894	A	50	3.6 ± 0.3
	B	113	3.4 ± 0.5
TREU 2034	C	168	4.1 ± 0.6
TREU 2037	D	168	4.2 ± 0.4
TREU 1885	E	36	4.7 ± 0.3
	F	108	5.4 ± 0.3
	F	168	4.1 ± 0.3
TREU 1881	G	59	6.0 ± 0.3
	G	80	5.8 ± 0.3
	H	124	4.8 ± 0.3
	I	64	4.0 ± 0.3

considered separately. From the four infectivity values for TREU 1881 and the three infectivity values for TREU 1885 it appears that the number of days in culture does not affect the infectivity of the metacyclic forms to laboratory mice. The values for infectivity vary markedly even within the same stock but a significant difference ($p = 0.05$) only exists if the difference in $\log_{10}$ ID_{63} /ml estimates equals or exceeds 1.2 (Lumsden et al, 1963).

3.7.3 Effect of the derivation of column purified metacyclic forms on the infectivity ($\log_{10}$ ID_{63}) values of TREU 1881

To determine what effect the derivation of the metacyclic forms had on their infectivity to mice, two populations of metacyclics were obtained, one from the supernatant directly and the

other after washing the culture forms attached to the flask substratum. Both populations were passed through DE52 cellulose anion exchange columns and the results of the infectivity titrations are presented in Table 3.9. The largest proportion of metacyclics, 87%, was obtained from the supernatant compared with 13% from the washed population. Hence, the greatest number of infective metacyclics with surface coats were free swimming in the supernatant at the time of harvest. However, the proportions of infective metacyclic trypanosomes obtained from both populations were similar, suggesting that there is no difference between the column separated metacyclic populations and that their derivation is not a factor in the low infectivity values obtained.

TABLE 3.9

Results of infectivity tests of DE52 separated metacyclic forms of T. congolense TREU 1881 from culture supernatant and from washed cultures.

Origin	Number of metacyclics ($\log_{10}$)	% of total metacyclics harvested	$\text{Log}_{10}\ ID_{63}/ml$[a]
Supernatant	7.6	87	4.8 ± 0.5[a]
Washed	6.7	13	4.6 ± 0.3

[a] from an initial concentration of $\log_{10}$ 7.0 metacyclics per ml

3.7.4 The development of local skin reactions in rabbits using in vitro-derived metacylic forms

The ability of in vitro-derived metacyclic forms of five of the Zambian T. congolense stocks, TREU 1885, TREU 1881, TREU 1894, TREU 2037 and TREU 2034 to produce local skin reactions of at least 5 mm in diameter in rabbits was determined (Table 3.10). Cultured

metacyclic forms of T. congolense TREU 1457 are known to consistently produce local skin reactions and this stock was included as a positive control on each rabbit. One of the three rabbits infected with TREU 1881 produced a reaction at day 10 post-infection with 10^2 metacyclics. However, the other two rabbits in that group only produced reactions with at least 10^3 metacyclics. All the stocks used produced local skin reactions with as few at 10^3 metacyclic forms. Fly induced infections with these Zambian stocks did not produce local skin reactions (data not presented), unlike tsetse flies infected with TREU 1457.

TABLE 3.10

Ability of in vitro-derived metacyclic forms of T. congolense to produce local skin reactions in rabbits.

Number of metacyclics	TRYPANOSOME STOCKS TREU 1885	TREU 1881	TREU 1894	TREU 2034	TREU 2037	TREU 1457
10^6	+	+	+	+	+	+
10^5	+	+	+	+	+	+
10^4	+	+	+	+	+	+
10^3	+	$+^2/3$	+	+	+	+
10^2	-	$+^1/3$	-	-	-	-

+ development of local skin reaction
- no reaction

3.7.5 Metacyclic production in vitro over a period of time

The absolute numbers of metacyclics and the proportion of metacyclic forms relative to other insect forms produced by five T. congolense cultures was examined over a fourteen day period involving six harvests. The results are shown in Figure 3.4 and Figure 3.5.

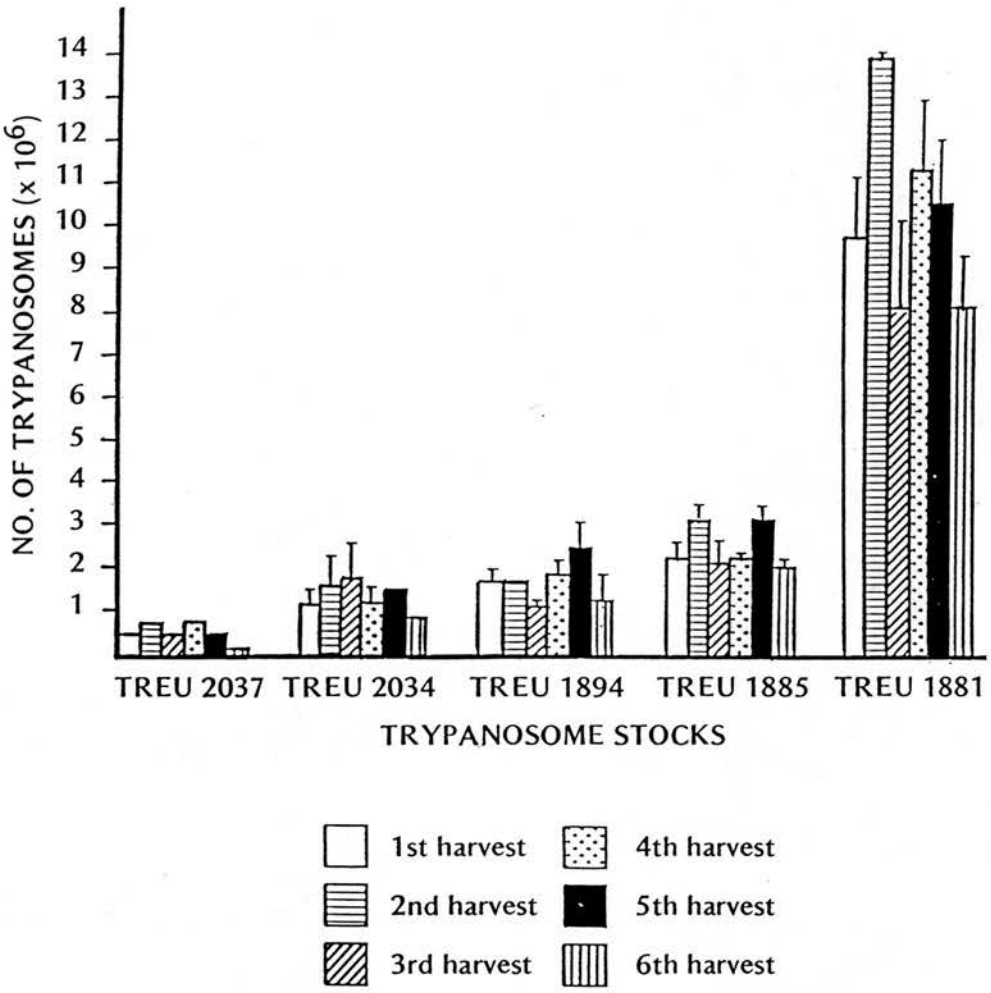


FIGURE 3.4

The number of metacyclic trypanosomes produced *in vitro* from *T. congolense*, TREU 2037, TREU 2034, TREU 1894, TREU 1885 and TREU 1881 over six consecutive harvests.

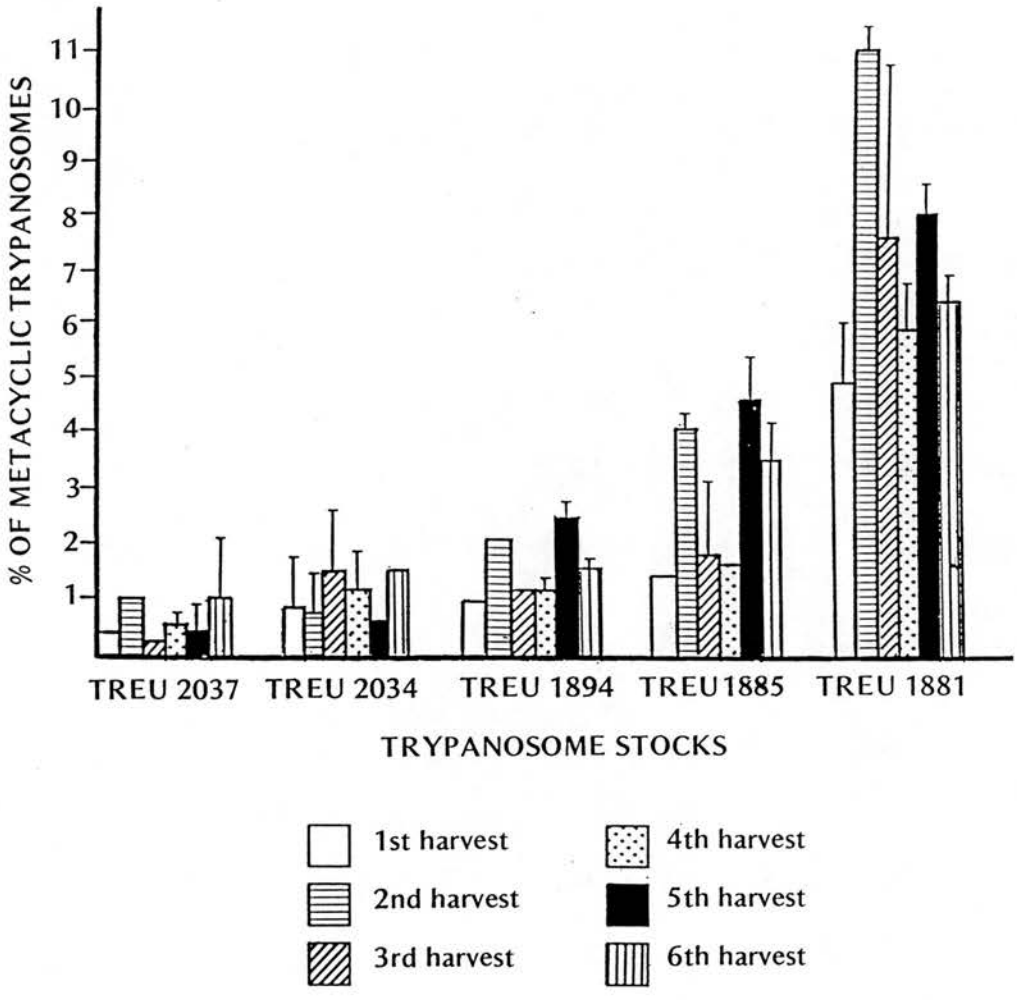


FIGURE 3.5

The relative proportions of metacyclic trypanosomes compared to other insect forms produced *in vitro* from *T. congolense* TREU 2037, TREU 2034, TREU 1894, TREU 1885 and TREU 1881 over six consecutive harvests.

Three of the stocks, TREU 1894, TREU 1885 and TREU 2034 produced between $1x10^6$ and $3x10^6$ metacyclics per flask on each harvest. TREU 2037 produced the lowest numbers of metacyclics at less than $1x10^6$ metacyclics per flask while TREU 1881 showed a marked increase over the other stocks producing at least $8x10^6$ metacyclics per flask. In general, the five stocks showed little day to day variation in the production of metacyclic forms although TREU 1881 did show a wide range of between $8x10^6$ and $1.4x10^7$ metacyclics per flask.

The proportions of metacyclic forms relative to other insect forms (Figure 3.5) show a similar pattern to the absolute numbers of metacyclics produced (Figure 3.4) in that TREU 2037 produced fewer metacyclics in proportion to other insect forms while TREU 1881 produced a much larger proportion. However, TREU 1894, TREU 1885 and TREU 1881 also showed a rise in the proportion of metacyclics to other insect forms on day two and day five which correspond to the second medium change after the 72 hour culture period. It is possible that with some stocks, the longer time interval between medium changes had an effect on the metacyclic production, relative to the other insect forms present.

3.8 DISCUSSION

The successful establishment of a T. congolense insect form culture producing animal infective metacyclic forms requires the attachment of epimastigotes to the surface of a flask or other suitable substratum. However, the presence of epimastigotes in the original isolation material is unnecessary; as demonstrated here, proventricular or midgut forms can differentiate in vitro to

epimastigotes. One advantage of using an infected proventriculus is that there are large numbers of trypanosomes present, increasing the probability of successful establishment of trypanosomes on the flask substratum (Gray et al, 1984).

Trypanosomes in the tsetse fly proboscis are exposed to strong mechanical forces caused by the incoming bloodmeal or secreted saliva (Thevenaz and Hecker, 1980). Therefore, attachment of the epimastigote stage probably prevents the trypanosomes from being dislodged during the feeding process and thus retaining the fly infection in a region where the metacyclic progeny are available to infect the vertebrate host. Alternatively, the attachment of epimastigote forms could possibly be the start of a series of processes leading ultimately to metacyclic production.

It appears that T. congolense epimastigote proliferation is not dependent on attachment. However, epimastigote attachment is a necessary requirement for metacyclic production and non-attachment imposes restrictions on the differentiation process (Hendry and Vickerman, 1988). There is a delay in metacyclic production of eight to 18 days after passage of trypanosomes to a new culture flask (Gray et al, 1987) and in the tsetse fly proboscis, epimastigotes are found several days before metacyclics are evident (Lloyd and Johnson, 1924; Hoare, 1972).

Although unattached epimastigotes are observed in culture supernatant it appears that they represent attached trypanosomes that have become dislodged during medium changes. Epimastigote forms have been observed in infected flies' salivary probes (Otieno and Darji, 1979) and if these forms were extruded with tsetse saliva into the

vertebrate host they would be rapidly killed by the host's immune response. However, unattached epimastigotes are known to differentiate to procyclic forms in vitro (Ross, personal communication) and if this was reflected in the in vivo situation it could represent a trypanosome survival mechanism where epimastigotes washed into the midgut during feeding could differentiate and maintain the infection within the fly. In practice this seems unlikely since flies which have mature infections in the mouthparts have been shown to be clear of infection in the gut. This could be related to factors in the host blood such as complement which may be lethal to the uncoated trypanosome in the tsetse fly midgut.

The variation in time taken for different trypanosome isolates to differentiate to epimastigotes and attach to the flask substratum (Tables 3.2 - 3.7) could be due to the way in which the cultures were manipulated and not necessarily be a reflection of the stock's ability to adapt to in vitro culture. The volume of supernatant and frequency of medium changes could both affect the rate of differentiation from procyclic forms to epimastigote and metacyclic trypanosomes. Trypanosome concentrations also affected the process, with some stocks tolerating a higher density of parasites per ml of culture supernatant than others. Conversely, if trypanosomes were allowed to increase to numbers which the medium could not support, then this could result in increased death.

In general, the use of Falcon Primaria flasks facilitated the successful establishment of infective cultures because of the increased adherence of epimastigote forms. These flasks have a positively charged substratum (Pittner, Fears and Brindley, 1985)

which probably aids the attachment of uncoated trypanosomes. However, there were disadvantages in using these flasks. Loose fitting caps led to the contamination of long term primary cultures which contained no antibiotics. In addition, although epimastigote attachment was good, mature bundles rapidly deteriorated, becoming swollen and granular, which led to an inability for those trypanosomes to be sub-passaged.

Figures 3.4 and 3.5 show a wide range in the numbers of metacyclics and the proportion of metacyclics to other culture forms. Gray et al (1984) also noted differences in the numbers of metacyclics produced from two stocks, one from East Africa and one from West Africa. They attributed this to a problem of adaptation to in vitro culture. However, as more stocks are cultured in vitro it appears that the wide range of morphological and biological characteristics displayed by T. congolense in the insect vector (Hoare, 1972; Stephen, 1986) are reflected when the trypanosomes are cultured in vitro. The wide variation in insect forms; procyclics, epimastigotes and metacyclics produced on different days even within the same stock could be attributed to the general handling of the cultures. The number of trypanosomes harvested in culture supernatants was a direct result of the treatment of the culture and depended on the strength of washing and the amount of material scraped from the flask bottom.

Morphologically, trypanosomes eluted from an anion-exchange column resemble metacyclic forms derived from infective tsetse flies. Results from the infectivity titrations are low for all stocks (Table 3.8). Gray et al, (1984) also reported proportions of infective trypanosomes of between 0.2% and 6.3% and suggested that the reasons for

this were that some metacyclics had incomplete surface coats, that the trypanosomes were damaged in the anion exchange column or that intraperitoneal infection of the metacyclics was in some way harmful to the trypanosomes. It has recently been demonstrated that separation of bloodstream forms of T. brucei on DEAE-cellulose columns at pH 8.0 causes marked effects on the parasite's biochemistry including a drop in intracellular ATP levels, and an increased release of VSG, peptidase and phospholipase (Lonsdale-Eccles and Grab, 1987). Although these trypanosomes appeared morphologically normal and they remained infective to mammalian hosts it is possible that biochemical changes to T. congolense metacyclics during anion exchange column separation could affect the trypanosomes' viability. Some of the biochemical changes were reduced by using Iscove's medium supplemented with adenosine, hypoxanthine, thymidine, pyruvate and mercaptoethanol. It was also suggested that trypanosome viability might also be improved if the separation was carried out at ambient temperatures rather than $4^{o}C$ which could have adverse effects on the morphological and biochemical aspects of the parasite's biology.

Intradermal inoculation of 10^{3} metacyclic forms of the five Zambian stocks of T. congolense cultured in vitro elicited local skin reactions in rabbits (Table 3.10); although fly induced infections with these stocks failed to produce local skin reactions (data not presented). Local skin reactions produced by in vitro-derived metacyclic forms have been reported previously in rabbits (Luckins, Rae and Gray, 1981) as well as cattle (Akol, Murray, Hirumi, Hirumi and Moloo, 1986) and goats (Dwinger, Lamb, Murray and Hirumi, 1987).

Local skin reactions were produced in Hereford cattle using 200 *in vitro*-derived metacyclics whereas 400 metacyclics were needed to cause smaller reactions in Boran (Akol *et al*, 1986). This suggests a relationship between host susceptibility and the ability of metacyclics to produce local skin reactions which would explain why larger numbers were required to produce such reactions in rabbits. In rabbits, for instance, 10^5 metacyclics from *in vitro* culture were required to achieve 100% infectivity and local reactions occurred within five days and reached a maximum severity six days post-infection (Dwinger *et al*, 1987).

The number of metacyclics extruded by a tsetse fly infected with *T. congolense* is thought to be between three and 350 (Harley and Wilson, 1968; Nantulya *et al*, 1980). Metacyclics produced from the *in vitro* culture systems described here range from 3.0×10^5 per flask to 1.4×10^7 per flask (Figure 3.4) depending on the trypanosome stock. Other workers have reported the production of *T. congolense* metacyclics as low as 2.0×10^5 per flask (Hirumi *et al*, 1982) to 1.2×10^7 per flask (Gray *et al*, 1984). *In vitro* cultures of *T.b rhodesiense* insect forms produce up to 1.0×10^6 metacyclics per flask (Kaminsky *et al*, 1987). This indicates that while the number of metacyclics produced appears to be stock dependent, larger numbers are consistently produced *in vitro* probably a result of the increased surface area to which the epimastigotes are attached compared to the tsetse salivary gland epithelium for (*T.b rhodesiense*) and the chitinous labrum in *T. congolense* infected flies.

Metacyclics produced *in vitro* from the Zambian isolates have facilitated the analysis of the antigenic characteristics of *T.*

congolense without having to infect large numbers of tsetse flies: cultured metacyclic forms have been used in the development of immunoassays to determine the serological relationships of these and other stocks isolated at the same time from the same geographical area.

CHAPTER FOUR

THE PRODUCTION AND USE OF MONOCLONAL ANTIBODIES TO METACYCLIC VARIABLE ANTIGENIC TYPES

4.1 INTRODUCTION

Animals have evolved an adaptive or acquired immune response which provides a flexible and specific reaction to infection. When the host encounters a foreign substance two different types of immunological reaction may occur: a humoral response involving B-cells giving rise to the synthesis and release of free antibody into the blood and body fluids and a cell mediated immune response involving T cells.

Antigens entering the body are trapped within lymphoid tissue and after intracellular processing, antigenic determinants are expressed on the cell surface in association with products of the Major Histocompatability Complex (MHC) class II genes. T-helper cells specific for the antigen proliferate after the recognition of the antigen-MHC class II complex. These cells then secrete a number of lymphocyte growth factors which stimulate the differentiation and proliferation of antigen activated B-cells as well as further division of T-cells. The expansion of both cell populations is clonal, but from each clone two distinct populations are produced: short-lived effector cells (plasma cells or cytotoxic T-cells) and long-lived memory cells. It is the presence of these memory cells that leads to the enhanced and accelerated secondary response (Roitt, 1988).

Antibody is a product of B-lymphocytes and plasma cells and is either cell bound or secreted as an extracellular product. There are five major classes of proteins or immunoglobulins associated with antibody, IgG, IgM, IgA, IgD and IgE and they can be differentiated from each other on the basis of biological function, physical or biochemical properties (Roitt, 1988). During B-cell activation and differentiation, the class of antibody expressed and secreted changes in a predetermined manner (Hobart and McConnell, 1975). The first immunoglobulin (Ig) class to be produced after infection is IgM but after activation of the plasma cells the expression of IgG subclasses or IgA or IgE begins (Bellanti, 1985).

4.1.1 Monoclonal antibodies

The manufacture of predefined specific antibodies by means of tissue culture cell lines resolved the disadvantages inherent in heterogeneous immune sera. Antibody producing B-cells have a limited life span and it is not possible to establish and maintain clones of such cells in vitro. However, myeloma cells can be cultured in vitro and these cells produce large amounts of immunoglobulins called myeloma proteins. Unfortunately, myeloma cells cannot be induced to produce antibody to a specific antigen (Köhler and Milstein, 1975).

Cell lines cultured in vitro which secreted anti-sheep red blood cell antibodies were produced by Köhler and Milstein (1975) by fusing mouse myeloma cells and spleen cells from a mouse immunized with sheep red blood cells. The resultant hybrid myelomas or hybridomas expressed both the splenic lymphocyte's property of specific antibody production and the immortal character of the myeloma cells. The

disadvantage of parent myeloma immunoglobulins being secreted along with the specific antibody derived from the parent B-lymphocyte was overcome by the preparation of non-secreting mutant myelomas. Using these cells, only immunoglobulin derived from the parent B-cell was secreted from the hybridomas produced from the fusion (Kearney, Radbruch, Liesegang and Rajewsky, 1979).

Since spontaneous cell fusion is rare, the two cell types need to be prepared under special conditions using a fusing agent. Sendai virus, lysolecithin and polyethylene glycol (PEG) have all been used successfully but PEG has been used most widely and is generally considered to be the agent of choice (Goding, 1980). The mechanism of fusion is poorly understood but it is thought that fusing agents operate by disrupting the cell membrane allowing the formation of heterokaryons which possess two or more nuclei. At the next division, the nuclei fuse and a hybrid cell results (Poste and Nicholson, 1978; Knutton, and Pasternak, 1979; White and Helenius, 1980). When B-cells from an immunized spleen are mixed with myeloma cells in the presence of PEG, fusion of the cells takes place randomly. In a mixture of fusion products and unfused cells, the number of myeloma cells is far greater than the number of hybridomas formed from the fusion. Also, the hybridomas are much slower growing at this stage and therefore a procedure is required to select the hybridomas secreting the desired antibody.

The selection of the desired hybridomas is achieved in three steps. Firstly, unwanted parent cells are removed from the fusion products using a selective growth medium in which only the fused hybridomas will survive. The hybridomas secreting the required

antibody are then identified in an appropriate screening assay and finally these cells are cloned, thus selecting a population of hybridomas secreting antibody of identical class and affinity to a single epitope.

In normal mammalian cells the main biosynthetic pathways for purines and pyrimidines can be blocked by the folic acid antagonist, aminopterin. However, cells can still synthesise DNA via salvage pathways in which preformed nucleotides are recycled and which depend on the enzymes thymidine kinase (TK) and hypoxanthine guanine phosphoribosyl transferase (HGPRT). Thus, if the cell is provided with thymidine and hypoxanthine DNA synthesis can still occur providing the enzymes TK and HGPRT are present (Littlefield, 1964). The myeloma cells used in fusions are mutants which lack HGPRT and therefore are unable to grow in medium containing aminopterin. When B-cells from an immunized spleen are fused with these mutant myelomas, they supply the resultant hybrids with HGPRT and therefore medium containing aminoprotein supplemented with hypoxanthine and thymidine (HAT medium) is used for the selection of hybridomas.

The second step in detecting hybridomas secreting the desired antibody requires a screening assay. This assay should select antibodies suited to the purpose for which they will eventually be used and also be able to screen many samples in a short period of time (McBride, 1983). The IFAT, enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) have the advantage of being rapid and are suitable for initial screening using small volumes of supernate. These tests can also be used to identify the class of

immunoglobulin, thus giving an indication of the functional property of the antibody.

The third step involves cloning the hybridomas selected by the screening assay to remove unwanted cells and variants not secreting antibody or producing irrelevant antibody which may overgrow the cells producing the required antibody. Periodic re-cloning is essential if the cells are kept in culture for many generations. Cloning can be achieved by several methods: one method uses limit dilution where cells are plated out in microtitre plates and serially diluted to give wells containing one cell. This method is most successful when feeder cells are used (Lernhardt, Andersson, Coutinho and Melchers, 1978; Levy, Dilley and Lampson, 1978). An alternative method of cloning hybridomas is to use soft agar (Coffino, Bamal, Laskov and Scharff, 1972; Pearson, Pinder, Roelants, Kar, Lundin, Mayor-Withey and Hewett, 1980). Colonies may be picked out of the agar by a finely drawn pasteur pipette. The cells are then grown in liquid medium and the supernatant tested for activity. In contrast, cloning by limit dilution allows direct testing from the wells containing the cloned cells. A third method of cloning involves the use of a fluorescence activated cell sorter (FACS) (Parks, Bryan, Oi and Herzenberg, 1979). The main advantage of using a FACS is that antibodies with identical specificity but with different biological properties can be selected. For example, using this technique hybridomas could be labelled with antigen-coated fluorescent latex microspheres and cloned on the basis of their antibody binding properties.

Typical IgG levels in culture supernatants are 10 µg/ml, compared to between 5 and 20 mg/ml in serum or ascites fluid (Galfre and Milstein, 1982) although IgM levels are usually lower (Goding, 1980). The production of ascites fluid in mice can be favoured by injecting the mice with pristane (2, 6, 10, 14-tetramethyl pentadecane) 7-10 days before injecting them with hybridomas (Hoogenraad, Helman and Hoogenraad, 1983)

4.1.2 The applications of monoclonal antibodies in trypanosomiasises

Three areas of research in trypanosomiasis where monoclonal antibodies have several advantages over polyspecific antisera are in serodiagnosis, analysis of the structure of VSG, and in examining the extent of M-VAT heterogeneity. The main advantage of monoclonal antibodies lies in their specificity for single antigenic determinants although their reproducibility and, in theory, their limitless supply compared to polyspecific antisera are also advantageous.

4.1.2.1 Serodiagnosis

In serodiagnostic tests, current assays suffer from a lack of specific, well defined antigen or antibodies (Nantulya, Musoke, Rurangirwa, Saigar and Minja, 1987). The Card Agglutination Trypanosomiasis Test (CATT) represents the most widely used successful test using VSG's (Magnus, Vervoort and Van Meirvenne, 1978). However, the complex nature of VSG expression means that the degree of specificity attained by this test is largely dependent on the selection of appropriate VSG's (Vervoort, Magnus and Van Meirvenne, 1983). Although this test is appropriate for *T. b. gambiense* which has a restricted VAT repertoire it is not successful for other

trypanosome species which have larger VAT repertoires. Using non-variant, common antigens in serological assays has had some limited success (Silayo, Gray and Luckins, 1980; Pearson *et al*, 1986). However, assays employing lysed trypanosomes show considerable cross reactivity with other species (Luckins, 1977) and other genera (Voller, Bidwell and Bartlett, 1975b). Another problem in assays involving the detection of anti-trypanosomal antibodies is that after chemotherapy, the antibodies persist thus complicating the assessment of the infection status of individuals (Luckins, Gray and Rae, 1978).

The detection of circulating trypanosomal antigens may be a more sensitive means of practical diagnosis and could enable the diagnosis of current infections. Rae and Luckins (1984) used hyperimmune sera to lysed trypanosomes in an ELISA to detect circulating antigens. However, the combination of antibody heterogeneity and cross reactions between common antigens made it difficult to identify the infecting trypanosome species. The specificity of this test was based on the relative absorbance values obtained in homologous and heterologous reactions.

Monoclonal antibodies have been produced using various immunization protocols which recognize *T. congolense* (Parish, Morrison and Pearson, 1985) and *Trypanozoon* subgenus (Richardson *et al*, 1986) procyclic stage specific antigens. *Trypanosoma* genus specific monoclonal antibodies have also been produced (Burgess and Jerrels, 1985). In addition, monoclonal antibodies raised against *in vitro*-derived procyclic forms of *T. congolense*, *T. b. brucei*, *T. b. rhodesiense* and *T. vivax* have recognized *Nannomonas* and *Trypanozoon* subgenus specific antigens and *T. vivax* species specific antigens

respectively in both procyclic and bloodstream form lysates (Nantulya et al, 1987). These antibodies and those described by Parish et al (1985) and Richardson et al (1986) could be used for the detection of infection in tsetse flies including the identity of the infecting trypanosome species. This could provide a useful epidemiological tool for the investigation of the interaction between the vector, parasite and host (Nantulya et al, 1987). Recently, a double antibody sandwich ELISA has been developed by Liu and Pearson (1987), to detect circulating antigen using the monoclonal antibodies prepared by Richardson et al (1986) to T. b. rhodesiense. It was discovered that these monoclonal antibodies were not procyclic stage specific as was first thought but also identified antigen on the bloodstream forms of T. b. brucei. Although the development of this assay remains at an early stage it does show a potential for the use of monoclonal antibodies as a diagnostic tool in the detection of trypanosomiasis.

Two monoclonal antibodies have been identified that react with a species specific antigen of T. cruzi irrespective of the strain or developmental stage of the parasite (Tachibana, Nagakura and Kaneda, 1986). When a competitive ELISA was carried out using one of these monoclonal antibodies, all sera from patients with Chagas' disease showed positive inhibition. In contrast, all patients with leishmaniasis or other parasitic diseases were scored as seronegative (Tachibana, Nagakura and Kaneda, 1988). This suggests that the competitive ELISA using this species specific monoclonal antibody will be useful in serodiagnosing Chagas' disease particularly in areas where leishmaniasis is co-endemic.

4.1.2.2 Analysis of VSG structure using monoclonal antibodies

The trypanosome surface coat provides the primary barrier between the host's immune system and the parasite and is also the site of antigenic variation (Barry and Vickerman, 1979; Vickerman and Luckins, 1969). A knowledge of the three dimensional structure of VSG's is essential for a proper understanding of the organization of the surface coat. Monoclonal antibodies allow a more precise definition of the relationships between epitopes than conventional antisera. It appears that epitopes identified with conventional antisera only represent those which are immunodominant and not those which are potentially immunogenic or biologically relevant (Hall and Esser, 1984). Five distinct antigenic determinants have been recognized from one cloned VSG from T. brucei although only one of those determinants was exposed on the surface of living trypanosomes (Miller et al, 1984a, b). In a study by Hall and Esser (1984) using 30 monoclonal antibodies, 14 reacted in immunofluorescence uniformly with both live and acetone-fixed trypanosomes, seven reacted with the flagellar pocket on live trypanosomes but uniformly on fixed trypanosomes and nine reacted only on fixed trypanosomes. All of the antibodies recognizing epitopes on live trypanosomes and three of those binding to the flagellar pocket were capable of neutralizing the infectivity of trypanosomes bearing the homologous VSG. It appears, therefore, that there are variant specific epitopes on the VSG molecule which are cryptic in the intact surface coat. This is possibly due to a combination of the conformation of each VSG molecule and the way in which they are arranged on the surface coat (Turner, 1985).

4.1.2.3 The examination of M-VAT heterogeneity using monoclonal antibodies

Using polyclonal antisera to metacyclic populations it was established that M-VATs were characteristic for any given serodeme (Le Ray et al, 1978; Jenni, 1977a). However, using this technique it was not possible to identify individual M-VATs. Since the immunizing metacyclics were antigenically heterogeneous the antiserum raised would be correspondingly heterospecific. However, by raising monospecific polyclonal sera to individual T. brucei M-VATs expressed on bloodstream form trypanosomes it was possible to determine that the T. brucei metacyclics extruded from infected tsetse flies were heterogeneous with respect to VAT (Le Ray et al, 1978; Gardiner, Jones and Cunningham, 1980). To determine the extent of M-VAT heterogeneity using this approach would involve cloning metacyclic forms and growing populations of trypanosomes all expressing the same VAT. This approach is not feasible for all species or many stocks of trypanosomes because of their lack of infectivity to laboratory animals.

The use of monoclonal antibodies with their epitope specificity would allow the selection of antibodies recognizing individual M-VATs from an inoculum of the entire M-VAT repertoire. The technique was used successfully to show the existence of M-VAT heterogeneity in T. b. brucei (Esser, Schoenbechler, Gingrich and Diggs, 1981; Crowe et al, 1981). Mice were immunized by repeated tsetse fly bite followed by drug therapy or by inoculation of irradiated metacyclic forms. Four M-VATs of one stock of T. b. rhodesiense were identified (Esser et al, 1981) whilst in another, two monoclonal antibodies identified

between 18 and 30% of one serodeme of T. b. brucei (Crowe et al, 1981). The M-VAT repertoire expressed by a clone of T. b. brucei was shown to be heterogeneous and limited in comparison to those expressed in the bloodstream of the mammalian host. Using six monoclonal antibodies, 80-90% of the metacyclic population extruded by infected tsetse flies was labelled (Nantulya, Musoke, Moloo and Ngaira, 1983).

Until recently the number of antigens constituting an M-VAT repertoire had only been defined for one stock of one trypanosome species, T. congolense TREU 1457 (Crowe et al, 1983). This investigation was greatly facilitated by the use of large numbers of antigenically stable metacyclic forms from insect culture systems (Gray et al, 1981; 1984). Twelve distinct M-VATs were identified by monoclonal antibodies using IFA and neutralization of infectivity tests. There were no differences between the M-VATs expressed by the T. congolense TREU 1457 in vitro-derived metacyclics and trypanosomes from TREU 1457 infected tsetse fly probes.

The M-VAT repertoire of a cloned stock of T. b. rhodesiense has also been examined by monoclonal antibodies. Twenty seven antibodies labelled 99.3% of the trypanosomes in a tsetse fly salivary probe. The small proportion of unlabelled parasites was thought to be uncoated trypanosomes. Successful neutralization experiments have indicated that the M-VAT repertoire of this stock of T. b. rhodesiense is limited to a maximum of 27 different VATs (Turner et al, 1988).

The large number of M-VATs observed by Turner et al (1988) contrasts with the relatively low number identified in T. congolense

. However,
(Crowe et al, 1983) there are few reports on the entire M-VAT repertoire in any species of trypanosome. The following experiments were undertaken to determine the number of M-VATs in a stock of T. congolense from Zambia, TREU 1885 and also in later experiments to examine whether some of those VATs were present in other isolates.

4.2 MATERIALS AND METHODS

4.2.1 Media

Fusion 1

RPMI 1640 was prepared in one litre batches from powder (Flow Laboratories, Irvine) and 25 mM HEPES buffer, 100 IU/ml penicillin and 100 µg/ml streptomycin were added before filtering through a 0.22 µm filter. The medium was dispensed into 100 ml bottles and stored at 4°C. This medium was defined as RPMI incomplete. RPMI complete medium was prepared by adding the following to the stock medium after filtering:

1 ml, 7% sodium bicarbonate

0.8 ml fungizone (250 µg/ml)

1 ml L-glutamine (200 mM)

50 µl 2-mercaptoethanol (0.1 M)

15 ml heat inactivated FBS

Fusions 2, 3 and 4

In Fusions 2, 3 and 4 liquid RPMI 1640 medium (Gibco, Europe) was used as the stock medium. RPMI complete medium was prepared in the same way as for Fusion 1 except for the addition of sodium bicarbonate which was already present in the stock medium.

4.2.2 Polyethyleneglycol (PEG)

PEG 6000 (BDH Chemicals) was used in two solutions PEG A and PEG B which were prepared as follows:

Solution PEG A 41.6% PEG/15% DMSO in RPMI 1640 incomplete medium.

20.8 g PEG was dissolved in warm incomplete medium and the solution prepared to a volume of 42.5 ml. This solution was autoclaved and 7.5 ml DMSO was added when the solution cooled.

Solution PEG B 25% PEG in RPMI incomplete medium

6.25 g PEG was dissolved in warm RPMI 1640 incomplete medium and the solution prepared to a final volume of 25 ml. This solution was autoclaved.

4.2.3 Selection medium

Hypoxanthine (13.611 g/l), thymidine (3.576 mg/l) and aminopterin (0.176 mg/l) (50X HAT) was supplied by Flow Laboratories, Irvine. Two ml of 50X HAT was added to 48 ml RPMI complete medium containing peritoneal macrophages.

4.2.4 Myeloma cell lines

The myeloma cell line X63-Ag8-653 (Flow Laboratories, Irvine) which does not synthesise or secrete immunoglobulin chains (Kearney et al, 1979) was used. Ten days before the fusion, cells were removed from liquid nitrogen and resuscitated by warming the cryopreservation tubes in a 37^{o}C water bath. The cells were then added to 8 ml RPMI incomplete medium and centrifuged at 200 **g** for five minutes at 4^{o}C. After centrifugation, the supernate was removed and the cell pellet resuspended in four ml RPMI complete medium. The

resuspended cells were then added to a T-25 tissue culture flask and placed in 5% CO_2 in air at 37°C. The cells were maintained at densities of $2.0x10^5$ to $1.0x10^6$/ml until the day of fusion.

4.2.5 Immunization protocol

Fusion 1

The immunization protocol was similar to that of Crowe et al (1983). A mouse was infected intravenously (i.v.) with $2x10^6$ in vitro-derived metacyclic forms of T. congolense TREU 1885. On day five post-infection, the animal was treated with 10 mg/kg diminazene aceturate (Berenil, Hoechst Ltd.). On day 14 post-infection the mouse was challenged with the same number of metacyclics i.v. The mouse was killed three days later and the spleen was removed for fusion.

Fusions 2, 3, and 4

The immunization protocol used in Fusions 2, 3 and 4 was similar to the protocol used in Fusion 1. However, the trypanosomes used to immunize the mice in Fusions 2 and 3 had been incubated at 4°C for 60 minutes with five monoclonal antibodies prepared in Fusion 1 to remove the M-VATs recognized by those monoclonal antibodies. In Fusion 4, the metacyclics were incubated with the nine monoclonal antibodies prepared previously in Fusions 1 and 3 before being inoculated into the mice.

4.2.6 Preparation of myeloma cells

On the day of fusion, myeloma cells in logarithmic growth phase were harvested and counted on an improved Neubauer haemocytometer. 10^7 myeloma cells were washed three times in ten ml RPMI incomplete medium and centrifuged at 200 **g** for five minutes at room

temperature (RT) between washes. After washing, the cells were resuspended in five ml RPMI incomplete medium and stored in a waterbath at 37°C.

4.2.7 Preparation of spleen cells from immunized mice

Method 1

This method was used in Fusions 1 and 2. The immunized mouse was killed by cervical dislocation, washed in 70% alcohol and its spleen removed aseptically. The spleen was placed in a petri dish with five ml RPMI incomplete medium and disrupted coarsely using two pairs of curved forceps. Larger clumps were allowed to settle for ten minutes. Homogenization was completed by repeatedly passing the cells through a series of hypodermic needles from 21 G to 26 G. Finally, the cells were washed three times in 10 ml RPMI incomplete medium, as in 4.2.6, then resuspended in 5 ml RPMI incomplete medium and stored in a waterbath at 37°C.

Method 2

Method 2 was used for Fusions 3 and 4. Preparation of the cells was similar to Method 1 except that there was no coarse disruption of the spleen. Five ml of RPMI incomplete medium was expelled from a syringe barrel through a 21 G hypodermic needle and into the intact spleen. The cell suspension was drawn back into the syringe and expelled through the hypodermic needle and spleen repeatedly using a series of hypodermic needles from 21G to 26G. The cells were then washed and stored using the same procedure described for Method 1.

4.2.8 Fusion of spleen cells and myeloma cells

10^7 myeloma cells in five ml RPMI incomplete medium were mixed with 10^8 viable spleen cells also in five ml RPMI incomplete medium in a 10 ml conical centrifuge tube. The mixture was centrifuged at 200 **g** for five minutes at RT. After complete removal of the supernate, 0.5 ml PEG A solution was added over a 30 second period using a one ml pipette. During this time the pellet was disrupted by the tip of the pipette and the cell suspension was mixed gently for another 30 seconds. A volume of 0.5 ml PEG B solution was added over a 30 second period and the pellet was further disrupted for two to three minutes with the pipette tip. Nine ml of RPMI incomplete medium was then added slowly over a ten minute period with a constant rocking of the tube. The cell suspension was then transferred to a 50 ml conical centrifuge tube before 40 ml of RPMI complete medium containing peritoneal macrophages from a Balb/c mouse was added over a further 10 minute period. The cells were plated out in microtitre plates by adding 100 µl of the cell suspension to each well. The plates were then incubated at 37^oC in 5% CO_2 in air. Twenty four hours after the fusion, 100 µl of RPMI complete medium supplemented with peritoneal macrophages (Fusion 1 and Fusion 2) or normal spleen cells (Fusion 3 and Fusion 4) and HAT was added to each well.

4.2.9 Selection of antibody secreting hybridomas

All the wells were examined microscopically for positive hybridoma growth. Seven days after fusion, all wells were given 75 µl of fresh medium. When the hybridoma cells in positive wells had become at least half confluent and the medium had not been changed

for 48 hours the supernate from those wells was removed and assayed for antibody by IFAT.

4.2.10 Testing for antibody production by the hybridomas using the indirect fluorescent antibody test

4.2.10.1 Antigen preparation

In vitro-derived metacyclic forms of *T. congolense* TREU 1885 were centrifuged at 600 *g* and resuspended at a concentration $>10^7$ trypanosomes per ml in PBS pH 8.0 containing 1% (w/v) glucose (PSG). These trypanosomes were placed on 15 well multitest slides (Flow Laboratories) in 10 µl amounts and air dried before being acetone-fixed for 10 minutes. After trypanosome fixation the slides were dried and stored in plastic bags at -40°C with a desiccating agent (silica gel).

Formalin-fixed trypanosomes were prepared by incubating the metacyclics in 1% (v/v) formalin in PBS pH 8.0 at 4°C overnight. The metacyclics were then washed three times in PBS to remove excess formalin before being resuspended in PBS at a concentration $>10^7$ trypanosomes per ml. The trypanosomes were added to 15 well multitest slides in 10 µl amounts, air dried and stored at -40°C with silica gel until used.

Unfixed metacyclic trypanosomes were used to determine whether the monoclonal antibodies recognized exposed epitopes on living trypanosomes. Trypanosomes were harvested from culture on the day of use and maintained at 4°C in PBS pH 8.0 throughout the procedure.

Culture-derived procyclic trypanosomes of TREU 1885 were acetone-fixed and stored in the same way as the metacyclic forms.

Uncoated procyclics were used as negative controls throughout the initial screening of hybridoma supernatants.

4.2.10.2 Screening procedure

Ten µl of each supernatant tested was placed in one well of prepared multitest slides which were then incubated in a humid chamber at RT for 30 minutes. After two washes in PBS pH 8.0 the slides remained in PBS for five minutes. The area around each well was dried and RAM/Ig/FITC (Nordic Laboratories) was added at a dilution of 1:80. After incubation for 30 minutes at RT in a humid chamber the slides were washed as before then mounted in a 60% glycerol/PBS solution. Trypanosomes were examined using a Leitz Orthoplan microscope equipped for incident light excitation of FITC using an I2 filter block and HBO 200 high pressure mercury vapour lamp. The proportions of fluorescing trypanosomes were then calculated by counting trypanosomes under transmitted light and then counting the number of trypanosomes fluorescing under incident light. At least 250 trypanosomes were examined per well.

Living metacyclic trypanosomes were prepared for fluorescence in 11x50 mm test tubes. The trypanosomes were first incubated in 100 µl of supernatant at $4^{o}C$ for 30 minutes. Before being washed three times in PBS pH 8.0. Each wash was followed by centrifugation of the trypanosomes at 600 **g** for 15 minutes at $4^{o}C$. After the final wash, the resuspended cells were incubated with RAM/Ig/FITC at a dilution of 1:10 at $4^{o}C$ for 30 minutes. There followed another three washes as before. The trypanosomes were then resuspended in PBS pH8.0 and examined by fluorescence microscopy.

4.2.11 Expansion of hybridoma cells from microtitre to multi-well plates

Confluent, antibody producing hybridomas were transferred from the 96-well microtitre plates to 24 or 48-well plates in order to facilitate cell growth. Cells to be transferred were resuspended in the well by gentle scraping and pipetting. One drop was replaced in the microtitre well and the remaining cell suspension was transferred to one of the wells of the 24 or 48 well plate to which medium containing mouse peritoneal macrophages had already been added. Medium changes were made at three to four day intervals until the cells became confluent and were cloned.

4.2.12 Cloning of hybridoma cells

Hybridomas in the 24 or 48-well plates were removed by gentle agitation by pasteur pipette. The viable cells were counted using an improved Neubauer haemocytometer and diluted 1:10 in RPMI complete medium. A concentration of 160 hybridoma cells per ml was then prepared and 0.9 ml of this cell suspension was added to 0.9 ml medium containing peritoneal macrophages to give a final concentration of eight cells per 100 µl. Serial two-fold dilutions were then carried out three times to give three cell suspensions of four hybridomas per 100 µl, two hybridomas per 100 µl and one hybridoma per 100 µl. The cells were then dispensed into the wells of 96-well microtitre plates as follows:

In column	1	eight cells per well in eight wells
	2	four cells per well in eight wells
	3	two cells per well in eight wells
	4	one cell per well in eight wells
	5	one cell per well in eight wells

After seven days, 100 µl of fresh medium was added to all wells and the wells examined for hybridoma growth. If single colonies appeared in any wells in columns four or five, they were marked and, when confluent, cells were assayed for antibody by IFAT. Cells which continued to secrete antibody were transferred to 24-well plates and maintained as described until they were re-cloned.

4.2.13 Preparation of ascites fluid from mice

Balb/c mice were treated with 0.5 ml pristane which was administered intraperitoneally. Seven to 10 days after pristane treatment, between 1 x 10^6 and 5 x 10^6 hybridomas in 0.5 ml RPMI complete medium were injected intraperitoneally into each mouse. Tumours developed and between 10 and 20 days after injection of the hybridomas, the fluid was drained from the peritoneum into 10 ml conical centrifuge tubes (Sterlin, UK) which were kept at 4°C on ice. After collection, the ascites fluid was centrifuged at 600 **g** for 15 minutes at 4°C. The supernatant was removed and stored at -40°C until used.

4.2.14 Determination of the immunoglobulin class of each monoclonal antibody

The immunoglobulin class of the antibody secreted by hybridomas was determined by IFAT as described previously. A slight modification of the test was necessary. RAM/IgG(Fc)/FITC or RAM/IgM (µ chain)/FITC (Jackson Immunoresearch) were used in place of RAM/Ig/FITC.

4.2.15 Cryopreservation of hybridoma cell lines

Cells growing in logarithmic phase were harvested and centrifuged at 200 g for 10 minutes at RT. They were then resuspended in RPMI complete medium at a concentration of 3.0 x 10^6 cells per ml and added to an equal volume of medium containing 15% (v/v) Dimethyl sulphoxide (DMSO) to give a final DMSO concentration of 7.5% (v/v). The cell suspension was dispensed into 1.8 ml cryopreservation tubes (Nunc) and cooled at -60°C overnight in the gas phase of liquid nitrogen. Each vial was then transferred to liquid nitrogen and stored at -196°C until needed.

4.2.16 Resuscitation of cryopreserved cells

Cells were brought to RT quickly by incubating the cryopreservation tube in a water bath at 37°C. They were added to 8 ml of RPMI incomplete medium and centrifuged at 200 **g** for 10 minutes at RT. After discarding the supernatant, the cells were resuspended in 4 ml of RPMI complete medium. The resuspended cells were then placed in a T-25 tissue culture flask and incubated at 37°C in 5% CO_2 in air.

4.3 RESULTS

4.3.1 The production of monoclonal antibodies recognizing metacylic antigens of T. congolense TREU 1885

Four fusions were carried out to produce monoclonal antibodies to metacyclics of T. congolense TREU 1885 and the results of those fusions are shown in Table 4.1. In Fusion 1, 148 wells (31%) contained hybridomas after HAT selection and of these, 43 wells (29%) secreted antibody which recognized metacyclic forms of TREU 1885.

Five hybridoma cell lines secreting antibodies which appeared to recognise different M-VATs were selected and cloned.

TABLE 4.1

The production of hybridomas secreting antibody recognizing metacyclic forms of T. congolense TREU 1885

Fusion	Myeloma cell-line	Method	% hybridoma growth	% hybridomas secreting antibody	Number of hybridomas selected and cloned
1	X63-Ag8-653	1	31	29	5
2	X63-Ag8-653	1	0	0	0
3	X63-Ag8-653	2	29	10	4
4	X63-Ag8-653	2	0	0	0

Fusions 2 and 4 did not produce hybridoma cells after HAT selection indicating that the cells had not fused However, Fusion 3 produced a similar proportion of hybridoma growth to that of Fusion 1. One hundred and thirty nine wells (29%) showed successful hybridoma production. Fourteen of these hybridomas secreted antibodies recognizing metacyclic surface antigens, about one third of the number of secreting cells in Fusion 1.

To ensure that each of the monoclonal antibodies produced from Fusion 3 recognized different M-VATs from those recognized by the antibodies produced in Fusion 1 supernatants from the positive hybrids were screened not only individually but also in conjunction with each of the five antibodies from the first fusion. Four cell lines from Fusion 3 produced antibodies which recognized different

M-VATs from those identified by antibodies produced in Fusion 1 and were therefore selected, cloned and characterized.

4.3.2 Characteristics of each of the nine monoclonal antibodies obtained from Fusion 1 and Fusion 3

Table 4.2 shows the characteristics of the nine monoclonal antibodies produced against TREU 1885 metacyclic trypanosomes from two fusions. Six antibodies were of the IgG class and three were IgM class immunoglobulins. The monoclonal antibodies were designated TREUM (Trypanosomiasis Research Edinburgh University Monoclonals) 1.1-1.5 (from Fusion 1) and TREUM 3.1-3.4 (from Fusion 3). All the monoclonal antibodies recognized antigens on the surface coat of living, formalin-fixed or acetone-fixed metacyclic trypanosomes of TREU 1885 but not acetone-fixed in vitro-derived procyclic forms of the same trypanosome stock.

There were variations in the proportion of metacyclics recognized by each individual monoclonal antibody when the same metacyclic population was examined on different occasions and between metacyclic populations prepared at different times. For example, TREUM 1.3 recognized from 9.1% to 22.2% with a mean of 18.8% and TREUM 1.5 had a range from 2.5% to 13.2%. However, with some monoclonal antibodies such as TREUM 3.2 there was very little variation in the proportion of the metacyclic population recognized.

A discrepancy existed between the actual proportion of metacyclics labelled by a pool of the nine monoclonal antibodies and the calculated proportions estimated by addition of the numbers of metacyclics recognized by individual monoclonal antibodies. Between 65.3% and 78.8% of the metacyclic population were labelled by the

TABLE 4.2

Designation, isotype and staining characteristics of each of the nine monoclonal antibodies selected

Monoclonal antibody designation	Class of immunoglobulin	% acetone fixed metacyclics recognised (with means in brackets)	Recognition of formalin fixed metacyclics	Recognition of viable meta-cyclics	Recognition of acetone fixed procyclics
TREUM 1.1	G	3.4-9.9(5.7)	+	+	-
TREUM 1.2	M	8.5-12.5(10.2)	+	+	-
TREUM 1.3	G	9.1-22.2(18.8)	+	+	-
TREUM 1.4	G	5.9-15.8(11.2)	+	+	-
TREUM 1.5	M	2.5-13.2(7.5)	+	+	-
TREUM 3.1	M	5.1-11.2(7.3)	+	+	-
TREUM 3.2	G	7.1-8.5(7.5)	+	+	-
TREUM 3.3	G	3.7-6.9(5.3)	+	+	-
TREUM 3.4	G	4.1-12.2(9.2)	+	+	-
Total pool	G/M	65.3-78.8	N.D.	N.D.	N.D.

+ positive fluorescence
- no fluorescence
N.D. not determined
means calculated from at least three counts

pool of monoclonal antibodies whereas the arithmetic addition of the proportions of monoclonal antibodies gave an expected value of approximately 84% recognition of the population.

4.3.3 Examination of the relationships between the epitopes recognized by each of the nine monoclonal antibodies

The relationships between the epitopes recognized by each of the nine monoclonal antibodies were examined using acetone-fixed trypanosomes in IFAT. The monoclonal antibodies were pooled together in pairs and the proportion of metacyclics stained with each mixture was compared with the proportions of the metacyclics recognized with the monoclonal antibodies constituting that mixture. The results are shown in Table 4.3 and the proportions of the metacyclic population recognized by each pool was generally greater than or equivalent to the sum of the proportions stained by the individual monoclonal antibodies. However, in some cases such as in mixtures of TREUM 3.2 and TREUM's 1.1, 1.2, 3.3 and 3.4; between TREUM 1.2 and TREUM 1.4 and between TREUM 1.3 and TREUM 1.5 there were no differences in the proportions of metacyclics stained by the pools and the proportions stained by the individual components of the mixture.

4.4 DISCUSSION

Two different protocols using the myeloma cell lines X 63-Ag8-653 were used to produce monoclonal antibodies to M-VATs of one cloned stock of T. congolense, TREU 1885 (Table 4.1). The myeloma cell line X 63-Ag8-653 does not synthesise or secrete immunoglobulin chains of its own and thus results in hybridomas which secrete only B-cell derived immunoglobulin (Kearney et al, 1979).

TABLE 4.3

Proportions of the metacyclic population, expressed as percentages, recognized by the monoclonal antibodies using IFAT and the examination of the relationships between individual monoclonal antibodies

TREUM	1.1	1.2	1.3	1.4	1.5	3.1	3.2	3.3	3.4
1.1	6.2								
1.2	26.1	8.5							
1.3	26.6	21.3	9.1						
1.4	20.9	13.7	29.2	15.8					
1.5	16.9	26.3	9.8	24.5	13.2				
3.1	18.4	N.D.	20.4	N.D.	18.3	7.3			
3.2	6.0	7.4	15.9	22.7	N.D.	12.8	7.1		
3.3	19.6	20.8	8.5	21.9	32.0	19.8	7.4	6.9	
3.4	N.D.	17.1	24.4	29.6	20.0	16.6	9.1	23.4	9.8

N.D. Not determined

The B-cells used in all fusions were obtained by disrupting the spleen of an immunized mouse. Preparing the spleen cells without coarse disruption of the spleen (method 2) was thought to be less traumatic than method 1 for the cells although >90% viability was achieved by both methods as determined by the trypan blue exclusion dye test. It appears from the results that neither method was considerably better than the other.

The crucial stage of the procedure appeared to be at the fusion of the myeloma cells and spleen cells. In Fusions 2 and 4 no hybridomas were detected after fusion indicating that the cells had not fused successfully. There are many reasons why hybridomas might not be produced at the time of fusion. Various investigators have explored the use of different molecular weight PEG, the importance of pH during fusion, the ratio of spleen cells to myeloma cells, different batches of serum and types of medium, thymocytes and other feeder cells (Melchers, Potter and Warner, 1978). However, none has

resulted in a significant increase in the absolute yield of recoverable hybrids per immunized spleen although care with each of these variables probably produces more reproducible results (Yelton, Margulies, Diamond and Scharff, 1980). In Fusion 1 and 2, peritoneal macrophages from a syngeneic mouse were used as a feeder layer and in Fusions 3 and 4 the feeder layer was comprised of normal spleen cells from a non-immunized mouse. Feeder cells are a necessary addition to the hybridoma cultures particularly at the cloning stage and hybrid cells may not grow well when cultured alone (Goding, 1980). Commonly used feeder cells include thymocytes (Lernhardt et al, 1978; Oi and Herzenberg, 1980), normal spleen cells (Levy et al, 1978) or peritoneal cells (Hengartner, Luzzati and Schreier, 1978). The exact function of feeder cells is not known but one role may be to reduce the toxicity of the plastic tissue culture tray (Goding, 1980).

The same immunization schedule was used for all four fusions. Mice were initially primed intraperitoneally by a population of metacyclic trypanosomes followed five days later by diminazene aceturate drug treatment. Fourteen days post-treatment the mice were challenged intravenously with a metacyclic population of the same stock. Three days after challenge the spleen was removed and the cells used for fusion. This protocol of an intial priming followed by a single boost is effective in generating antibody producing hybridomas (Oi, Jones, Goding, Herzenberg and Herzenberg, 1978). The fact that the antigen was cell bound and that the metacyclics established an infection would also favour this protocol since cell bound surface antigens are highly immunogenic (Goding, 1980; Crowe, 1984). Also, intravenous challenge three days prior to fusion stimulates pre-B

lymphocytes to differentiate into cells which will form antibody secreting hybridomas (Crowe, 1984; Oi et al, 1978). Conversely, soluble proteins are often very poorly immunogenic in aqueous solution. When purified VSG is used to immunize mice it is usually necessary to use an adjuvant and a long immunization schedule (Reinwald, Greiser-Wilke, Artama, Risse and Molling, 1987; Lyon, Pratt, Travis, Doctor and Olenick, 1981).

Each of the nine monoclonal antibodies showed a variation in the proportion of the metacyclic population that it identified. This phenomenon has been reported before with two other unrelated stocks where although the M-VATs present in a population remained stable, the relative proportions of those M-VATs varied markedly over a period of time (Luckins et al, 1986; Prain and Ross, 1988). It is unlikely that this variability is due to errors incurred during counting or that the number of metacyclics counted in an individual sample might have been insufficient to make an accurate assessment of M-VAT proportions. Prain and Ross (1988) considered the possibility that the variation observed in the frequency of appearance of different M-VATs within the population could have been due to errors incurred during counting. They considered two methods of analysis; counting 200-500 metacyclics by fluorescence microscopy and counting a larger sample of between 10^5 and 10^6 metacyclics by flow cytometry thus increasing the accuracy of subpopulation determinations. Both methods gave similar results regardless of the disparity in sample sizes. It is also unlikely that the variation in proportions of metacyclics labelled is caused by the selective activation of individual VSG genes at the pre-metacyclic stage (Tetley et al, 1987).

Therefore, the variation observed with each monoclonal antibody in the proportions of the metacyclic population labelled is probably a consequence of variability intrinsic in the differentation process (Tetley et al, 1987; Prain and Ross, 1988). When pooled, the nine monoclonal antibodies recognized between 65% and 79% of the entire metacyclic population whereas when the individual values of the proportions stained by those monoclonal antibodies were added,at least 84% of the metacyclic population was recognized (Table 4.2). This anomaly has been reported previously (Crowe et al, 1983) and there are a number of possible explanations to account for it. Firstly, it could be due to examining different metacyclic populations obtained on different occasions. Secondly, individual trypanosomes could be expressing more than one VAT and thirdly, cross-reacting epitopes are present on different M-VATs. Steric hindrance of the binding of monoclonal antibodies which recognized determinants close together on the VSG molecule might also occur.

Variation in the proportions of M-VATs expressed in different populations does exist and has been discussed previously. However, in this case variation occurred even when all the trypanosomes had been obtained from the same metacyclic population. Although the presence of more than one VAT has been observed in bloodstream form trypanosomes (Esser and Schoenbechler, 1985) there is evidence to suggest that in T. brucei metacyclic forms each metacyclic VSG gene has a discrete activation (Tetley et al, 1987). The control of M-VAT expression in T. congolense is probably similar to that of T. brucei and it is therefore unlikely that more than one M-VAT could be expressed on the same trypanosome at the same time.

The possibility that cross reacting epitopes were present on different VATs is supported by the results shown in Table 4.3. In some cases there was no addition of the proportions of metacyclics stained when two monoclonal antibodies were pooled. Several studies have shown that many monoclonal antibodies which bind specifically VSGs and which are positive in immunofluorescence on air dried or acetone-fixed trypanosomes do not react with living trypanosomes (Pearson et al, 1980; Olenick, Travis and Garson, 1981). However, the nine monoclonal antibodies used here each recognized epitopes on live metacyclic trypanosomes. It has been shown that monoclonal antibodies defined as recognizing surface epitopes could show a limited overlap with antibodies recognizing cryptic determinants (Hall and Esser, 1984). If this is so then steric hindrance of binding of monoclonal antibodies recognizing epitopes adjacent on the VSG molecule would occur since a typical antigenic determinant comprises seven amino acids and the molecular weight of IgG is around 150,000 (Miller et al, 1984a).

It appears that the monoclonal antibodies produced here were not all recognizing different or unrelated epitopes because the proportion of the metacyclic population recognized by a pool of all the monoclonal antibodies did not concur with the expected proportion recognized when the values for the individual monoclonal antibodies were added independently. This could be due to steric hindrance of binding due to the recognition of adjacent epitopes on the VSG. Nevertheless, these monoclonal antibodies recognizing around 80% of the TREU 1885 metacyclic population confirm that the trypanosome metacyclic population is heterogeneous with respect to VAT and the results are consistent with those of Crowe et al (1983), in estimating the size of the M-VAT repertoire.

CHAPTER FIVE

EXAMINATION OF THE M-VAT SPECIFIC HOST IMMUNE RESPONSE BY ELISA

5.1 INTRODUCTION

5.1.1 The humoral immune response of the host to trypanosome infection

Infections with pathogenic trypanosomes result in a varied and complex immune response. The highly immunogenic antigenic determinants on the VSGs which are exposed on the surface of living trypanosomes elicit variant specific neutralizing antibodies. These antibodies appear within seven to 14 days of infection and have been shown to be related to remissions in parasitaemia (Boreham, 1968; Wilson and Cunningham, 1972). Common or stable antigens are components such as structural proteins or enzymes of membranes and organelles and produce a weaker antibody response than the variant antigens. These antigens may be found in different stocks of the same species, in different trypanosome species and some persist throughout the life cycle of the trypanosome (Le Ray, 1975; Gray and Luckins, 1976).

Studies on bovine and murine trypanosomiases have demonstrated marked increases in the concentration of serum IgM (Seed, Cornille, Risby and Gam, 1969; Luckins, 1972; Luckins, 1976; Hudson, Byner, Freeman and Terry, 1976; Kobayashi and Tizard, 1976) and IgG (Musoke, Nantulya, Barbet, Kironde and McGuire, 1981; Masake, Musoke and Nantulya, 1983; Luckins, 1972) although reports vary on the extent

of the increases. A characteristic feature of infections with all species of pathogenic trypanosomes studied is the hypergammaglobulinaemia which occurs soon after infection (Luckins, 1976; Clarkson, Penhale, Edwards and Farrell, 1975). This pathological defect could be a result of polyclonal B and T cell activation so that cells become refractory to selection by antigen and thus the antigen specific immune responses of the host become defective (Hudson et al, 1976). Studies in the rabbit concluded that little of the IgM produced during infection was parasite specific (Houba, Brown and Allison, 1969) and that much of this immunoglobulin class was largely comprised of non-trypanosomal antibodies, mainly autoantibodies directed against host components (MacKenzie and Boreham, 1974). In contrast, in T. brucei infected cattle, it has been shown that most of the IgM produced during the early stages of infection could be absorbed using a wide range of VATs (Musoke et al, 1981). However, sera from chronic infections were not examined during these experiments and it is possible that non-specific IgM could be produced in the later stages of infection. IgM is more effective than early IgG at neutralizing, agglutinating and lysing trypanosomes in vitro and providing protection in vivo (Seed, 1972; Takayanagi and Enriquez, 1973; Luckins, 1976; Musoke et al, 1981) although VAT specific antibody is present in both IgM and IgG fractions.

In experimental infections of cattle using syringe-passaged cloned populations expressing only one VAT, recurrent peaks of antibody against the infecting clones have been observed (Nantulya et al, 1979; Musoke et al, 1981; Musoke et al, 1983). This suggests

that there was a reappearance of the infecting organisms or of trypanosomes with variable surface antigens similar to those of the infecting clones. Cattle infected with cloned bloodstream T. brucei or T. congolense have shown complete immunity against tsetse-transmitted challenge with the homologous but not a heterologous stock after elimination of the trypanosomes from the peripheral blood circulation (Nantulya et al, 1984). Sera obtained from these animals before challenge contained neutralizing antibodies against metacyclic VATs of the homologous but not a heterologous stock. These results indicate that some of the bloodstream VATs that arise during the course of a chronic infection possess surface epitopes in their variable surface glycoproteins that are identical to those of the metacyclic VATs. Therefore, in some chronic infections it is possible that the VAT repertoire of the infecting trypanosomes could be exhausted, thereby leading to both 'self-cure' and subsequent immunity to homologous cyclically transmitted challenge.

5.1.2 Detection of anti-trypanosomal antibodies

Serological tests either measure the secondary effects of a primary antigen-antibody reaction or more directly, the capacity of antibodies to bind to an antigen. Most of the classical serological tests have been used in bovine trypanosomiasis including immune lysis (Dar, 1972), complement fixation (Mehlitz and Deindl, 1972; Lotzsch and Deindl, 1974) and various agglutination tests (Gray, 1967; Binz and Allsop, 1972; Robson, 1972). These tests measure secondary effects of a primary antigen-antibody reaction. Measuring the capacity of antibodies to bind to antigen is a more reliable method

of immunoassay. Three such binding assays are IFAT, RIA and ELISA and all of these tests detect less than 1µg of antibody per dl (Stites, Stobo, Fundenberg and Wells, 1982).

5.1.3 Principles of ELISA for antibody detection

In an ELISA for the detection of antibody, the antigen is attached to a solid phase support to allow for the separation of reacted and unreacted reagents. Microplates have been readily accepted as a solid phase since they permit batch processing of large numbers of sera.

The next stage involves the addition of the sera which are usually diluted to prevent non-specific reactions. The major reason for high non-specific reactivity is sample components that stick directly to the coated plastic surface. To reduce this, Tween 20 is often used in the serum diluent and in some systems it might be necessary to include an initial blocking agent such as bovine serum albumin (BSA).

After washing to remove excess unbound antibody, the conjugate, which is generally an anti-species antibody linked to an enzyme, is then added. For most purposes, horseradish peroxidase (HRPO) is a suitable choice of enzyme although in some assays, where the antigen has peroxidase activity, alkaline phosphatase is a good alternative to HRPO (Schuurs and Van Weemen, 1977). HRPO is cheap and readily available in purified form, it can be conjugated to antibody by several methods and there is a good variety of chromogenic substrates available.

After a further wash, the enzyme substrate is added. Chromogenic substrates are initially colourless and on enzymatic degradation produce a coloured soluble product. The amount of colour developed is proportional to the bound conjugate and hence the antibody content of the test serum. In colourometric determination of HRPO, orthophenylene diamine (OPD), 2,2'-azino-di-[3-ethyl-benzthiazoline-sulphonate] (ABTS), 5-aminosalicylic acid (5-ASA) and 3,3',5,5'-tetramethyl-benzidine (TMB) have all been used as hydrogen donors (Bos, Van der Doelen, Van Rooy and Schuurs, 1981). It is usual to permit substrate incubation for a given time and then stop the enzyme reaction by means of acid or alkali and to measure the absorbance of the solution of the substrate product (Voller and Bidwell, 1986).

Enzyme immunoassays can be classified as homogeneous or heterogeneous. Homogeneous assays are those in which the enzyme in the labelled reagent behaves differently depending on whether or not it is bound to its specific counterpart in the immune reaction and which therefore do not require a physical separation of the reactants into two fractions. Assays in which the label behaves identically, irrespective of whether or not it is bound to its specific counterpart in the immune reaction and which therefore require a separation of the reactants into two fractions are called heterogeneous enzyme immunoassays.

The major difference between ELISA and RIA, two heterogeneous assays, is the use of an enzyme to label the antigen or antibody rather than the less convenient radioactive isotope (Clark and

Engvall, 1981). Although, ELISA is slightly less sensitive than RIA (Stites et al, 1982) it uses simpler methods with relatively cheap, easily acquired reagents and equipment. Also, enzyme coupled reagents are generally stable during prolonged storage, thus eliminating the need for repeated labelling with isotopes which undergo decay and avoiding the dangers and special precautions involved in working with radioactive isotopes (Stocker et al, 1981).

5.1.4 Applications of the antibody detection ELISA to trypanosomiasis

Initially ELISAs for the detection of serum antibodies used polystyrene tubes coated with antigen (Engvall and Perlmann, 1972). A microscale modification of this technique was used to measure antibody responses in patients infected with Chagas' disease (Voller, Draper, Bidwell and Bartlett, 1975a). This microELISA had several advantages over the previous system. It was much less cumbersome and many serum samples could be assayed in replicate on one plate using small quantities of antigen, antibody and enzyme conjugates.

Several microELISA tests have been used in the serodiagnosis of South American and African trypanosomiasis and the results have been satisfactory both in terms of sensitivity and specificity (Voller et al, 1975a, 1975b; Ruitenberg and Buys, 1977; Luckins, 1977). However, severe cross-reactions were reported between trypanosomes of different species and with other kinetoplastids such as Leishmania. The reason for those cross-reactions could be explained by the methods used for preparing the trypanosomal antigen. The assays all used undefined soluble antigens from lysed trypanosomes prepared by

hypotonic lysis (Voller et al, 1975a; Ruitenberg and Buys, 1977) or sonication (Voller et al, 1975b; Luckins, 1977), thus exposing common antigens.

The presence of similar predominant VATs in taxonomically and geographically different populations belonging to the sub-genus Trypanozoon (Gray and Luckins, 1976; Van Meirvenne et al, 1977) gave new prospects of achieving species specific and VAT specific diagnosis of African trypanosomiasis (Vervoort, Magnus and Van Meirvenne, 1978). A practical diagnostic test was produced by purifying a commonly occurring VSG from bloodstream forms of T. b. gambiense isolated from distinct geographical areas throughout West and Central Africa. However, by purifying the VSG there is the probability that a loss of conformation of the glycoprotein will occur, thus allowing epitopes on regions of homology on the VSG and the cross reacting determinant to be exposed, thereby giving rise to cross reactions. These regions of the VSG are not normally exposed on the surface of living trypanosomes. Therefore, because of the presence of common somatic antigens, regions of homology on different VSGs and the common reacting determinant, the integrity of the trypanosome and the conformation of the surface coat should always remain as similar to that of a live trypanosome as possible when VAT specific responses are examined.

5.1.5 Preparation of cells for examination of surface antigens by ELISA

5.1.5.1 Assays involving cells in suspension

Although ideally it is best to use living cells when examining cell surface antigens, assays involving living cells in suspension are complicated by the need for freshly prepared viable cells. Such assays have cumbersome washing procedures between stages of preparation and mechanical disturbance of the cells can occur because of repeated centrifugation and resuspension (Heusser, Stocker and Gisler, 1981). However, by formalin fixing trypanosomes for IFAT (Jenni, 1977a) and glutaraldehyde fixing trypanosomes for RIA (Musoke et al, 1982; Morrison, Black, Paris, Hinson and Wells, 1982), assays examining cell surface variant specific antigens have been carried out successfully in suspension.

5.1.5.2 Attachment of cells to the solid phase

Assays performed with attached cells have several advantages over tests performed with cells in suspension; the washing procedure is rapid and efficient, the cells do not need to be repeatedly centrifuged and resuspended, therefore removing the possibility of mechanical disturbance. This may explain the improved reproducibility of assays involving attached cells. Morever, improved reproducibility allows the detection of low affinity and/or low quantitites of antibodies that may be directed against a minor population of cell surface antigens (Heusser et al, 1981).

Cells have been attached to plastic using several methods. Plates can be treated with heterologous antibodies directed to cell surface components (Barker, Worman and Smith, 1975; Stocker and

Heusser, 1979) or with lectins such as phytohaemagglutinin (PHA) (Stocker, Malavasi and Trucco, 1981). These anchoring methods have intrinsic disadvantages under some assay conditions. Coating the plate with antibodies requires the preparation of a specific antiserum which may contain added specificities that interfere with the test. Furthermore, enzyme-linked protein A cannot be used as a developing agent because it would bind to the antibodies used to coat the plates. The coating by lectins on the other hand requires special quenching procedures to saturate the sugar reactive sites of the lectin which in the case of PHA has been achieved using egg white glycoproteins (Stocker et al, 1981).

Immobilization of cells by glutaraldehyde fixation is the most common method of attaching mammalian cells to plastic (Stocker and Heusser, 1979; Fairchild and Moorhead, 1985; Kurita, Kiyono, Michalek and McGhee, 1985). It is a rapid and convenient method and results in stable fixed cells suitable for storage at $4^{o}C$. Glutaraldehyde has been used successfully as a tanning agent and probably works as a cross-linking agent (Fein and Filachione, 1957). It has been reported that glutaraldehyde can alter some cell surface antigens (Baron, Wernet, Schunter and Wigzell, 1977; Haaijman, Deen, Krose, Zylstra, Collen and Radle, 1984) and also that it can produce high background in ELISA due to non-specific binding (Lansdorp, Astaldi, Oosterhof, Janssen and Zeijlemaker, 1980). However, it has been used without adverse effects with bloodstream forms of T. brucei (Black, Hewett and Sendashonga, 1982; Musoke et al, 1981; Morrison et al, 1982) and T. congolense (Masake et al, 1983) in RIA where the cells remained

firmly attached to the plastic and also retained the surface antigens displayed on living trypanosomes.

The use of poly-L-lysine (PLL) to pre-treat ELISA plates has enhanced the attachment of mouse immunocytes and was shown to circumvent the use of cell fixatives (Epstein and Lunney, 1985). Although the mechanism of attachment is unclear, cell coated plates were found to be stable to storage for many months, suggesting that PLL acted as a mild fixative.

The aim of the experiments described in this Chapter was to produce an ELISA which was M-VAT specific using intact *in vitro*-derived metacyclic forms of *T. congolense*. The optimal method of antigen preparation was determined in relation to the numbers of metacyclics required, their attachment and fixation to the solid phase and storage of fixed metacyclics. Using the M-VAT specific ELISA, the immune response of the host to the entire M-VAT repertoire of *T. congolense*, TREU 1457 was examined.

5.2 MATERIALS AND METHODS

5.2.1 Solutions and reagents

5.2.1.1 Washing solution

The washing solution was used to remove excess fixative, antibody and conjugate from the wells. One litre of washing solution contained

sodium chloride	8 g
potassium dihydrogen orthophosphate	0.2 g

sodium hydrogen orthophosphate dodecahydrate	2.9 g
potassium chloride	0.2 g
Tween 20.	0.5 ml

5.2.1.2 Blocking solution

Ten grammes per litre of bovine serum albumin (BSA) fraction V (Sigma Chemicals Ltd) was added to the ELISA washing solution above (5.2.1.1). This solution was used to block reactive sites on the ELISA plates to reduce non-specific binding of the reagents.

5.2.1.3 Substrate buffer

A 0.5 M solution of citric acid was added to 500 ml of 0.6 M sodium acetate until a pH6.0 was obtained. The volume of this solution was adjusted to 600 ml with distilled water. This gave a five times concentrated solution and was diluted 1:5 in distilled water to give a 0.1 M sodium acetate/citric acid buffer.

5.2.1.4 Substrate

Ten mg of 3,3'5,5' - tetramethylbenzidine (TMB) was dissolved in one ml of dimethylsulphoxide (DMSO) and this solution was added to 99 ml of the substrate buffer (5.2.1.1). Fifteen µl of hydrogen peroxide (30% w/v) was added to the solution immediately before it was used.

5.2.1.5 Sera

Serum samples were collected from rabbits which had been inoculated intradermally with 10^5 *in vitro*-derived metacyclic forms or bitten with up to five tsetse flies infected with *T. congolense*. The samples were collected from each rabbit at seven day intervals

starting from day 0. Three rabbits were infected with each trypanosome stock.

T. congolense TREU 1457, TREU 1881 and TREU 1896 were used to provide metacyclic forms to prepare the ELISA plates and to raise the antisera which were used in the assay.

5.2.1.6 Conjugates

Goat anti-rabbit immunoglobulin (GAR/Ig) conjugated to horseradish peroxidase (HRPO) (Nordic Laboratories) was used at a dilution of 1:2000. Goat anti-rabbit IgG conjugated to HRPO (GAR/IgG/HRPO) (Jackson Immunoresearch) was used at a dilution of 1:4000. To determine the immunoglobulin isotype response a double labelling method was used with GAR/IgG (Cappel) or GAR/IgM (Nordic Immunologicals Ltd.) diluted 1:4000 or 1:800 respectively. The second step in this method used rabbit anti-goat immunoglobulins conjugated to HRPO (RAG/Ig/HRPO) at a dilution of 1:2000.

5.2.2 Optimization of plate preparation

5.2.2.1 Determination of the optimal numbers of metacyclics used to coat each well

The optimal number of metacyclics required to coat each well was determined by adding 10^4, 10^5 or 10^6 in vitro-derived metacyclics of T congolense TREU 1881 to the wells of 96-well flat bottomed polystyrene microELISA plates (Dynatech Laboratories). Twenty four wells per plate were used for each concentration of metacyclics and two plates were prepared for the test. The metacyclics were added to the wells in 50 µl of PSG pH8.0 and centrifuged at 100 g for 30 minutes at 4°C. After centrifugation, the trypano-

somes were fixed to the wells by adding 50 µl of a 0.5% (v/v) solution of glutaraldehyde to each well giving a final concentration of 0.25% (v/v). To remove excess fixative, the plates were washed three times in washing solution. After each wash, the washing buffer was flicked from the wells and the plates were allowed to stand in fresh washing solution for three minutes during each wash. Each well was then filled with 150 µl of washing solution and the plates were covered and stored at $4^{o}C$ for 24 hours. For each of the three concentrations of metacyclics, eight wells per plate per serum sample were used.

5.2.2.2 The effects of formalin and glutaraldehyde fixation of metacyclics on the results of the ELISA

Glutaraldehyde and formalin fixation of metacyclics were compared using two stocks of T. congolense, TREU 1457 and TREU 1881. Two plates per stock were prepared, one plate for each method of fixation and 10^{4} metacyclics per well were used for each stock. For formalin fixation, a 2% (v/v) formalin solution in 50µl was added to the trypanosomes after centrifugation at 100 g for 30 minutes at $4^{o}C$. The trypanosomes were fixed overnight at $4^{o}C$ before being washed and stored at described previously (5.2.2.1). Glutaraldehyde fixation was exactly as described above (5.2.2.1).

For the ELISA, NRS, anti-TREU 1896, anti-TREU 1881 and anti-TREU 1457 21 day post-infection rabbit sera were then added in duplicate to the wells in row A of the ELISA plate at a dilution of 1:200 and using serial two-fold dilutions, titrated down the plate to give a final dilution of 1:25,600 in row H.

5.2.2.3 The effects of time on glutaraldehyde fixation of metacyclics

Empirical testing of the effects of time on glutaraldehyde fixation of metacyclics was carried out using three time periods, one, five and ten minutes. 10^4 metacyclics of TREU 1896 in 50µl PSG pH8.0 were added to each well of two ELISA plates, centrifuged as before (5.2.3.1) and then fixed in 0.25% glutaraldehyde. On each plate, three columns (24 wells) were washed three times in washing solution after one minute fixation, another three columns were washed after five minutes and the last three columns, washed after ten minutes fixation. Serial two fold dilutions for NRS and anti-TREU 1896 (homologous) and anti-TREU 1457 (heterologous) 21 day post-infection rabbit antisera were carried out for each of the different fixation times on the two plates.

5.2.2.4 Pretreatment of ELISA plates with poly-L-lysine

Plates were treated with poly-L-lysine (PLL) at ten µg per ml in distilled water for two hours. The PLL was then flicked from the wells and the plates were allowed to dry overnight at RT. The following day, 10^4 metacyclic trypanosomes of T. congolense TREU 1881 were added to the wells in the normal manner and either fixed in 0.25% glutaraldehyde or not fixed before being used.

5.2.3 Test procedure

The procedure for the ELISA was the same throughout all of the tests following the plate preparations described previously. The plates were cleared of the washing buffer in which they were stored before 150 µl blocking solution was added to each well. After incuba-

tion at 37°C for one hour, the blocking buffer was removed. 100 µl of the diluted test serum was then added to each well and incubated at 37°C for 30 minutes. The plates were then washed three times with three minutes incubation in washing buffer between each wash. The enzyme conjugate was added and the plates were incubated again at 37°C for 30 minutes. When incubation with the conjugate was complete, the plates were washed three times as before. Addition of 100 µl of substrate was followed by another incubation at 37°C for 20 minutes. The reaction was stopped by adding 50 µl of 2 M H_2SO_4 to each well. Finally, the optical density (OD) of the colour reaction was read at 450 nm using a Titertek Multiskan ELISA plate reader (Flow Laboratories).

5.2.4 The effects of storage of prepared plates on ELISA values (E_{450}^{nm})

Two plates were prepared on the same day from the same population of TREU 1457 metacyclics. 10^4 trypanosomes were used to coat each well, the plates were centrifuged at 100 g for 30 minutes at 4°C then the metacyclics were fixed in 0.25% glutaraldehyde for ten minutes. After washing,the plates were covered and stored at 4°C. One plate was removed after 24 hours and used in the ELISA and the second plate was removed after seven days. Serum samples were taken at five day intervals from a rabbit infected with TREU 1457 metacyclics from day 0 to day 75 post-infection. The sera were diluted to 1:3200 and four serum samples were used per plate.

5.2.5 Optimal dilutions of GAR/IgM, GAR/IgG and RAG/Ig/HRPO

It was necessary to alter the standard ELISA protocol described above (5.2.3) to measure the IgG and IgM antibody response to the host. This modification involved adding either GAR/IgM or GAR/IgG in place of the GAR/Ig/HRPO. The conjugate was also changed from GAR/Ig/HRPO to RAG/Ig/HRPO. Chequerboard titrations were carried out using 10^4 glutaraldehyde fixed T. congolense TREU 1457 metacyclics per well as the antigen, 21 day post-infection anti-TREU 1457 serum for the IgM test and 35 day post-infection anti-TREU 1457 serum for the IgG test. NRS was used as a control in both chequerboard titrations. The assays were carried out as shown in Figure 5.1 for IgM test and Figure 5.2 for IgG test. Four wells were prepared for each second antibody and conjugate dilution.

5.2.6 Procedure for IgG and IgM specific ELISA

5.2.6.1 The IgM specific ELISA

10^4 glutaraldehyde-fixed metacyclics of T. congolense TREU 1457 were used to coat each well of two ELISA plates. Anti-TREU 1457 rabbit sera from day 0 to day 70 post-infection from three rabbits were used at a dilution of 1:3200 and replicate wells were used for each serum sample. A control of anti-TREU 1896 rabbit sera from day seven to day 56 post infection was included in the assay. The plates were incubated at 37°C for 30 minutes before being washed as described previously (5.2.3). After the third wash, GAR/IgM was added to each well at a dilution of 1:1000 and the plates were incubated for a further 30 minutes at 37°C. The plates were washed three times and

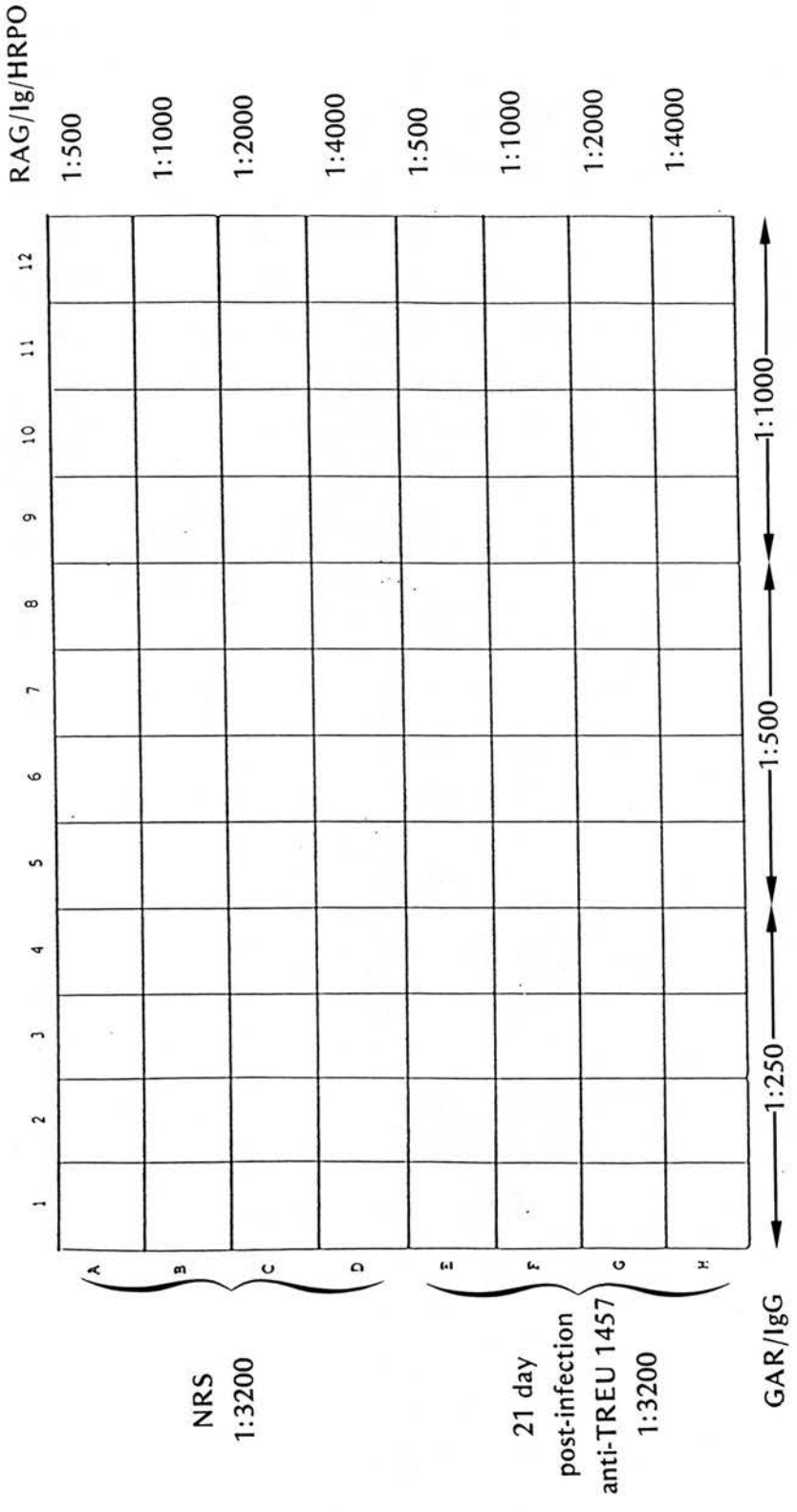


FIGURE 5.1

Chequerboard titration for the IgM test.

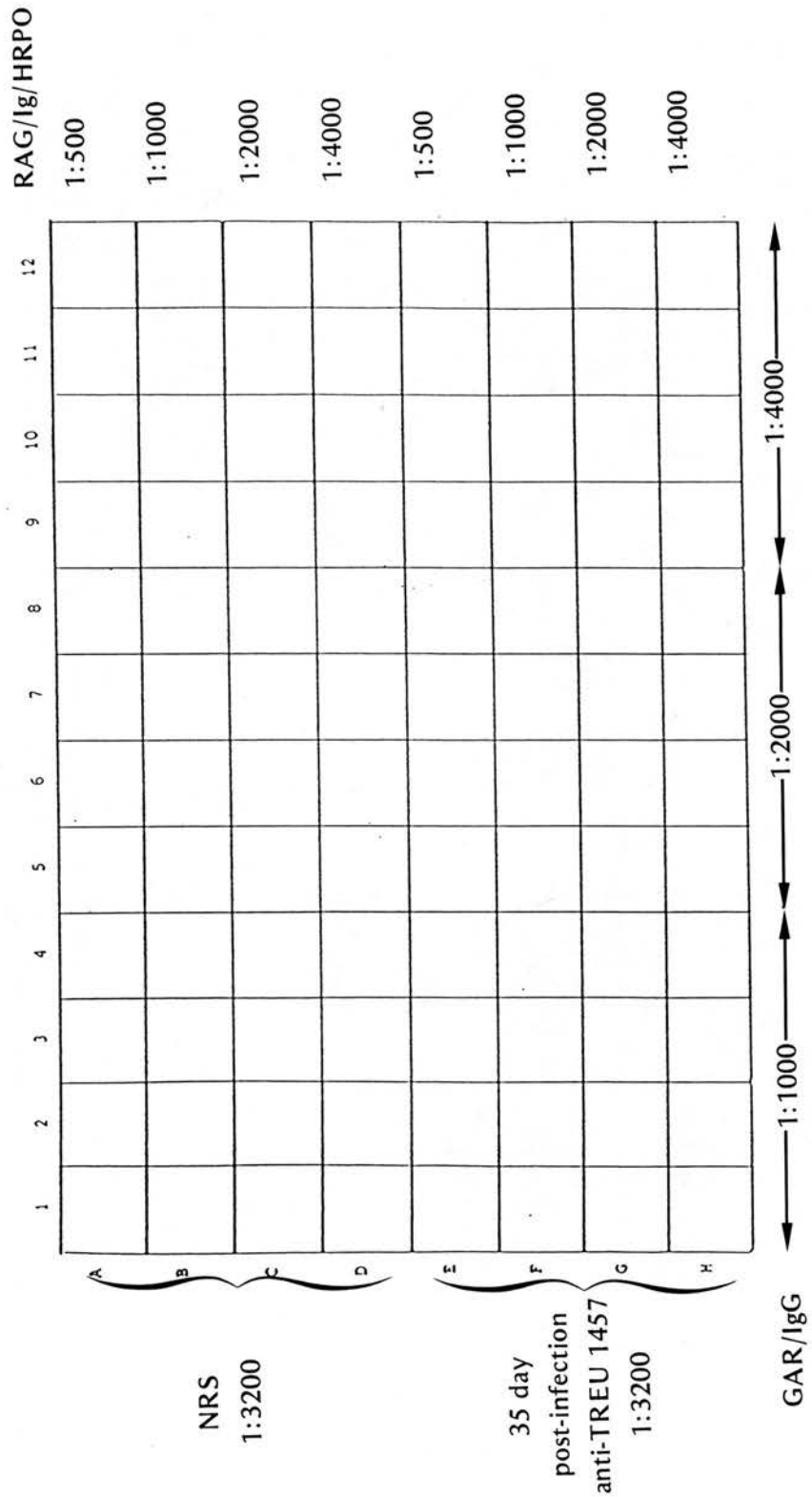


FIGURE 5.2

Chequerboard titration for the IgG test.

then RAG/Ig/HRPO was added at a dilution of 1:4000 and incubated at $37^{o}C$ for 30 minutes. The plates were washed before the substrate was added and the plates incubated at $37^{0}C$ for 20 minutes and then the reaction was stopped with 2M H_2SO_4 and the absorbance read at 450 nm.

5.2.6.2 The IgG specific ELISA

The procedure for this assay was essentially the same as that for the IgM assay (5.2.6.1). GAR/IgM was replaced by GAR/IgG which was used at a dilution of 1:2000.

5.3 RESULTS

5.3.1 The effect of different numbers of metacyclics per well on absorbance values (E_{450} nm)

10^4, 10^5 and 10^6 metacyclics of *T. congolense* TREU 1881 were used to coat the wells of ELISA plates to determine if variation in the number of organisms had an effect on absorbance values in ELISA tests. Individual results (Table 5.1) show little variation between wells and between plates as indicated by small standard deviations. Different numbers of metacyclics had no effect on the absorbance values obtained for the NRS controls. The ELISA values obtained for heterologous antiserum decreased as the numbers of metacyclics used to coat each well was increased. Using 10^6 metacyclics, the ELISA value for heterologous antiserum was equivalent to the ELISA value for NRS. The results for the homologous antiserum did not show such a distinct pattern, with the highest ELISA value being obtained by using 10^4 metacyclic trypanosomes. This ELISA value was

TABLE 5.1

The effect of different numbers of T. congolense TREU 1881 in vitro-derived metacyclics per well on absorbance values (E_{450}nm) as determined by ELISA using NRS, 21 day post-infection anti-TREU 1881 (homologous) serum and 21 day post-infection anti-TREU 1457 (heterologous) serum.

Number of metacyclics per well	ELISA values E_{450}nm ± S.D. *SERUM		
	NRS	21 day post-infection anti-TREU 1457	21 day post-infection anti-TREU 1881
10^4	0.084 ± 0.003	0.150 ± 0.006	0.386 ± 0.003
10^5	0.068 ± 0.006	0.114 ± 0.014	0.196 ± 0.002
10^6	0.065 ± 0.005	0.024 ± 0.006	0.242 ± 0.006

*All sera used at a dilution of 1:3200

also obtained during a subsequent experiment (Table 5.2) using 10^4 metacyclics of the same stock TREU 1881. Although the ELISA value for the positive heterologous control was equivalent to the ELISA value for NRS when using 10^6 metacyclics per well, the greatest difference between the ELISA values for homologous and heterologous antiserum were obtained when using 10^4 metacyclics per well. Using 10^6 metacyclics per well would require 10^8 metacyclics for each prepared plate which was impracticable, considering the numbers of metacyclics produced from the in vitro cultures. Therefore, because significant differences between ELISA values for homologous and heterologous antisera were found and practicable numbers required for the prepara-

TABLE 5.2

ELISA values after fixation of TREU 1881 and TREU 1457 metacyclics by formalin or glutaraldehyde.

			$E_{450}^{nm} \pm$ S.D.			
			ANTISERUM			
Fixative	Antigen	NRS	TREU 1881	TREU 1457	TREU 1896	TREU 1627
Formalin	TREU 1881	0.032 ± 0.012	0.150 ± 0.004	0.213 ± 0.000	0.104 ± 0.005	0.139
Formalin	TREU 1457	0.027 ± 0.003	0.050 ± 0.008	0.179 ± 0.034	0.092 ± 0.004	0.075 ± 0.011
Glutaraldehyde	TREU 1881	0.048 ± 0.002	0.292 ± 0.056	0.122 ± 0.006	0.125 ± 0.000	0.055 ± 0.011
Glutaraldehyde	TREU 1457	0.012 ± 0.012	0.022 ± 0.009	0.558 ± 0.004	0.070 ± 0.012	0.000 ± 0.000

tion of each plate, 10^4 metacyclics per well were used to prepare each ELISA plate.

5.3.2 The effects of fixative and of fixation time on ELISA values

To determine whether formalin or glutaraldehyde would be the fixative of choice for the *in vitro*-derived metacyclics, a comparison of the two fixatives was made using trypanosomes of two stocks, TREU 1881 and TREU 1457 at concentrations of 10^4 metacyclics per well (Table 5.2). ELISA values for the NRS control serum were unaffected by the method of fixation. Formalin fixation of TREU 1457 metacyclics showed considerably higher ELISA values for the homologous antiserum than the ELISA values for the three heterologous antisera used. However, TREU 1881 metacyclics which had been fixed in formalin produced ELISA values which indicated that cross reactions with heterologous antisera had occurred. The ELISA value for TREU 1457 antiserum was higher than the ELISA value for the homologous anti-TREU 1881 serum. Glutaraldehyde fixation of both TREU 1881 and TREU 1457 produced distinct differences in the ELISA values for homologous and heterologous reactions. As with formalin fixation, those differences were greater when using TREU 1457 metacyclics as the antigen than when using TREU 1881 metacyclics. Therefore glutaraldehyde was chosen as the fixative of choice for subsequent ELISA tests.

Although ten minutes fixation in glutaraldehyde produced ELISA results which showed little evidence for cross reactions between different stocks (Table 5.2), empirical testing of the effects of

glutaraldehyde fixation time was required. Three fixation times were employed and the effects on the subsequent ELISA values are shown in Table 5.3. The time of fixation with glutaraldehyde did not affect NRS control ELISA values which remained at the same level. The ELISA values for heterologous antiserum, although higher than the NRS control values remained lower than the ELISA values for the homologous reactions at each of the three fixation times. The lowest heterologous antiserum ELISA value and the highest homologous antiserum ELISA value were obtained after fixation of the parasites for ten minutes and it was concluded optimal conditions for glutaraldehyde fixation were 0.25% (v/v) for ten minutes at RT.

5.3.3 The effects of storage of prepared plates on ELISA values

The stability of glutaraldehyde-fixed metacyclics after short term storage was determined with plates prepared using TREU 1457 metacyclics. Two plates were stored at $4^{o}C$ for 24 hours and another two plates were stored at $4^{o}C$ for seven days. The two sets of results (Figure 5.3) were similar although in general, the ELISA values from the plate stored for seven days were higher, particularly with the sera from 35 days post-infection onwards. The pattern of antibody response was not affected by the storage time of the glutaraldehyde fixed metacyclics.

5.3.4 The effect of poly-L-lysine pre-treatment of plates on absorbance values

The effect of poly-L-lysine pre-treatment of ELISA plates with or without subsequent glutaraldehyde fixation on metacyclic

TABLE 5.3

Glutaraldehyde fixation of T. congolense TREU 1896 metacyclics: the effect of different fixation times on the absorbance values (E_{450nm}) as determined by ELISA using NRS, anti-TREU 1896 day 21 post-infection serum and anti-TREU 1457 day 21 post-infection serum.

Fixation time (minutes)	ELISA values $E_{450}{}^{nm} \pm$ S.D. *SERUM		
	NRS	21 day post-infection anti-TREU 1896	21 days post-infection anti-TREU 1457
1	0.024 ± 0.009	0.285 ± 0.003	0.228 ± 0.072
5	0.028 ± 0.000	0.352 ± 0.000	0.246 ± 0.001
10	0.031 ± 0.020	0.420 ± 0.031	0.168 ± 0.003

* All sera used at a dilution of 1:3200

trypanosomes was examined and the results are shown in Table 5.4. Homologous antiserum ELISA values were low with the poly-L-lysine treated plate although there were considerable differences between those and ELISA values for the heterologous antiserum. The largest differences between homologous and heterologous antisera ELISA values were observed with the plates prepared using the standard method of glutaraldehyde fixation although distinct differences were also observed with the pre-treated plates in which the metacyclics were subsequently glutaraldehyde fixed. Since poly-L-lysine pre-treatment of plates did not produce a distinct improvement in the results of the ELISA, the standard method of glutaraldehyde fixation was retained.

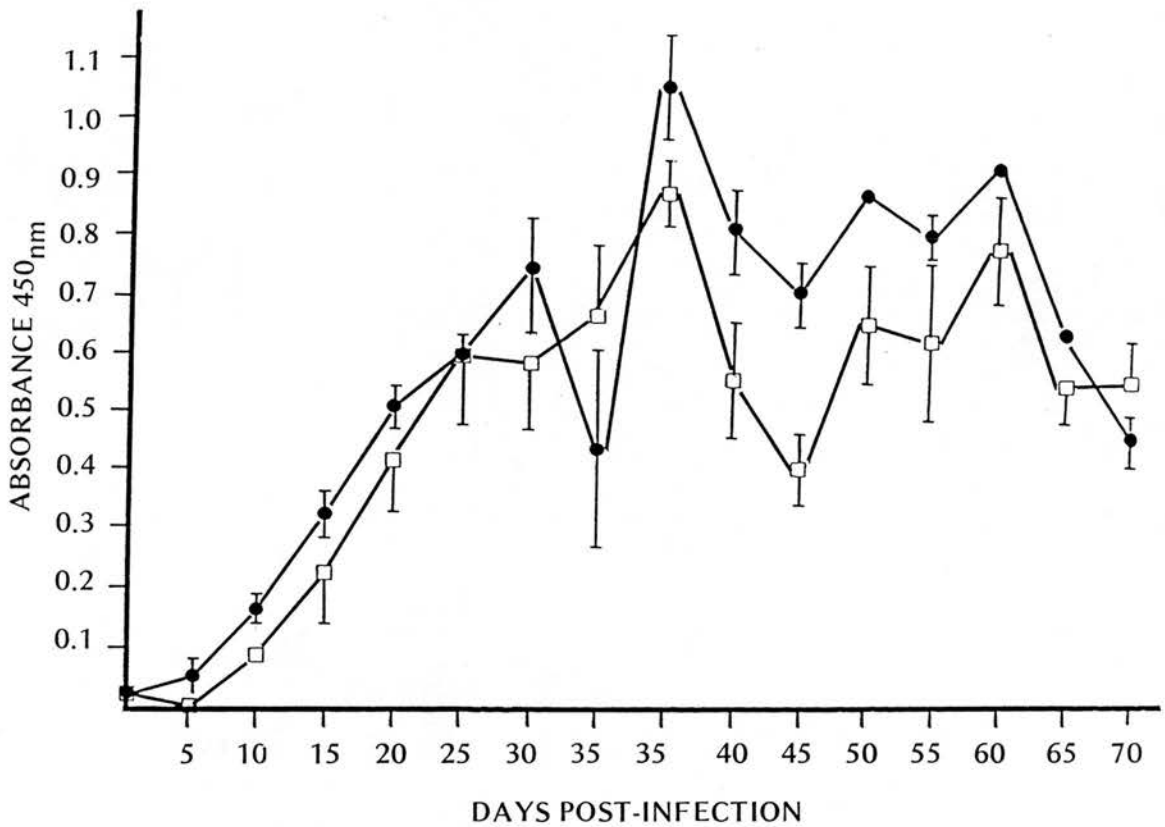


FIGURE 5.3

The effects of plate storage for one day (□——□) or seven days (●——●) on ELISA values using *T. congolense* TREU 1457 *in vitro*-derived metacyclics as antigen and anti-TREU 1457 rabbit serum used at a dilution of 1:3200.

TABLE 5.4

Comparison of the effects of pre-treatment of the plates with poly-L-lysine followed by subsequent glutaraldehyde fixation, poly-L-lysine treatment alone and glutaraldehyde fixation without pre-treatment by poly-L-lysine on the results of an ELISA using T. congolense TREU 1881 in vitro-derived metacyclics as antigen, NRS, 21 day and 35 day anti-TREU 1881 (homologous) serum and 21 day and 42 day anti-TREU 1457 (heterologous) serum.

Serum (1:3200 dilution)	ELISA values E_{450}nm ± S.D.		
	poly-L-lysine	glutaraldehyde	poly-L-lysine plus glutaraldehyde
NRS	0.216 ± 0.046	0.182 ± 0.022	0.008 ± 0.008
21 day post-infection anti-TREU 1881	0.240 ± 0.122	0.656 ± 0.062	0.498 ± 0.024
21 day post-infection anti-TREU 1457	0.082 ± 0.024	0.106 ± 0.046	0.036 ± 0.012
35 day post-infection anti-TREU 1881	0.168 ± 0.009	0.704 ± 0.024	0.688 ± 0.028
42 day post-infection anti-TREU 1457	0.072 ± 0.028	0.112 ± 0.090	0.228 ± 0.014

5.3.5 Determination of optimal dilutions of GAR/IgG, GAR/IgM and RAG/Ig/HRPO conjugate

The results of assays to determine optimal dilutions of GAR/IgM and RAG/Ig/HRPO are presented in Table 5.5. A chequerboard titration was carried out using GAR/IgM at dilutions of 1:250, 1:500 and 1:1000, 1:2000 and 1:4000. Two sera were used in this assay; a NRS control and a 21 day post infection anti-TREU 1457. When the conjugate was used at a dilution of 1:500 ELISA values for NRS were higher than ELISA values for the homologous antiserum. This was also the case when the conjugate was used at dilutions of 1:4000 and GAR/IgM was used at 1:500 and 1:1000. The ELISA values for the NRS

TABLE 5.5

Determination of the optimal working dilutions of GAR/IgM in conjunction with RAG/Ig/HRPO conjugate using T. congolense TREU 1457 metacyclics as antigen with NRS and 21 day post-infection anti-TREU 1457 rabbit serum.

Dilution of GAR/IgM	*Serum	ELISA values E_{450}nm ± S.D. Conjugate dilution 1:500	1:1000	1:2000	1:4000
1:250	NRS	0.402 ± 0.076	0.372 ± 0.081	0.397 ± 0.071	0.322 ± 0.015
	anti-TREU 1457 day 21 post-infection serum	0.347 ± 0.072	0.542 ± 0.103	0.451 ± 0.032	0.347 ± 0.013
1:500	NRS	0.402 ± 0.029	0.330 ± 0.016	0.21 ± 0.006	0.254 ± 0.020
	anti-TREU 1457 day 21 post-infection serum	0.287 ± 0.045	0.370 ± 0.064	0.288 ± 0.048	0.213 ± 0.007
1:1000	NRS	0.308 ± 0.088	0.239 ± 0.025	0.185 ± 0.019	0.140 ± 0.016
	anti-TREU 1457 day 21 post-infection serum	0.178 ± 0.018	0.264 ± 0.023	0.181 ± 0.018	0.125 ± 0.017

* All sera used at a dilution of 1:3200

control were lowest at a dilution of 1:1000 for GAR/IgM in conjunction with RAG/Ig/HRPO at 1:4000 and therefore, these dilutions were used for the subsequent IgM assay.

The results of the chequerboard titration to determine the optimal working dilutions of GAR/IgG and RAG/Ig/HRPO conjugate are shown in Table 5.6. At conjugate dilutions of 1:500, 1:1000 and 1:2000 the background levels as indicated by NRS ELISA values were high. When the RAG/Ig/HRPO conjugate was used at a dilution of 1:4000, the ELISA values for NRS fell to suitable background levels. The greatest difference between NRS ELISA values and those of the homologous antiserum were obtained when GAR/IgG was used at a dilution of 1:2000 and the conjugate diluted to 1:4000. These dilutions were used in the subsequent assay to determine the IgG response of the host to metacyclic trypanosomes of T. congolense TREU 1457.

5.3.6 The immune response of the host to T. congolense TREU 1457 metacyclics

The results of the IgM assay using homologous antisera are shown in Figure 5.4a. There was a rise in absorbance values from day zero to day seven post-infection for sera from all three animals tested. However, after day seven post-infection the patterns of absorbance values differed. In rabbit 1, ELISA values declined sharply by day 21 post-infection and thereafter progressively increased to day 70 post-infection. Serum samples from rabbit 2 maintained the ELISA values at the level observed on day seven post-infection through to day 21 post-infection and then showed a progressive fall to day 56 post-infection. ELISA values for rabbit 3

TABLE 5.6

Determination of the optimal working dilutions of GAR/IgG in conjunction with RAG/IgG/HRPO conjugate using T. congolense TREU 1457 metacyclics as antigen with NRS and 35 day post-infection anti-TREU 1457 rabbit serum.

Dilution of GAR/IgM	*Serum	ELISA values $E_{450}^{nm} \pm$ S.D. Conjugate dilution 1:500	1:1000	1:2000	1:4000
1:1000	NRS	0.202 ± 0.067	0.204 ± 0.026	0.170 ± 0.009	0.106 ± 0.010
	anti-TREU 1457 day 35 post-infection serum	0.427 ± 0.047	0.302 ± 0.023	0.276 ± 0.037	0.241 ± 0.017
1:2000	NRS	0.311 ± 0.020	0.149 ± 0.011	0.147 ± 0.013	0.102 ± 0.013
	anti-TREU 1457 day 35 post-infection serum	0.382 ± 0.051	0.305 ± 0.028	0.230 ± 0.016	0.252 ± 0.019
1:4000	NRS	0.280 ± 0.038	0.163 ± 0.025	0.361 ± 0.042	0.090 ± 0.000
	anti-TREU 1457 day 35 post-infection serum	0.186 ± 0.041	0.228 ± 0.012	-	0.206 ± 0.019

* All sera used at a dilution of 1:3200

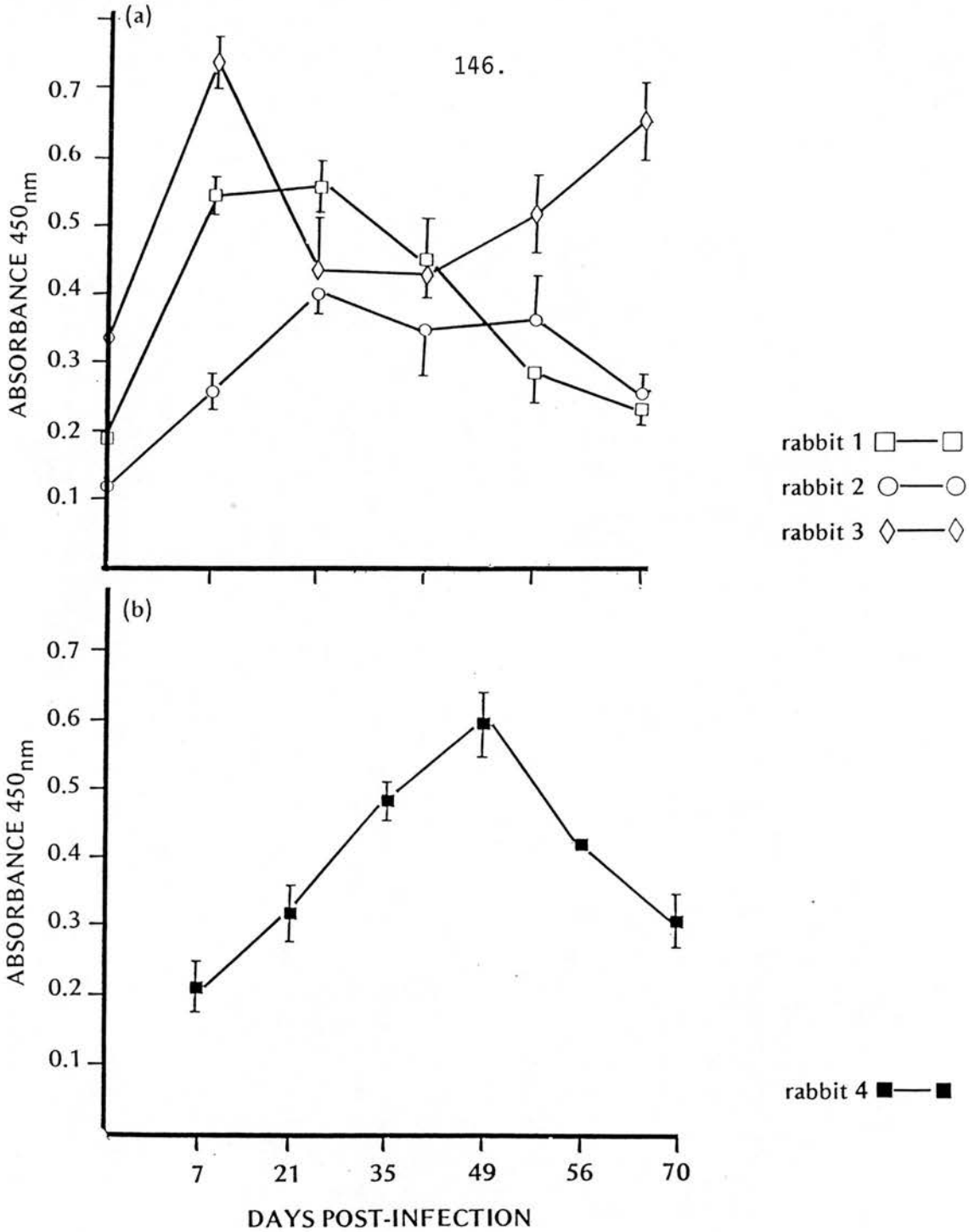


FIGURE 5.4

Examination by ELISA of the host's IgM response to TREU 1457 metacyclics.

(a) homologous response
(b) heterologous response

maintained their day 21 post-infection level through to day 56 post-infection and thereafter fluctuated through to day 70 post-infection. These results contrast with those obtained using anti-TREU 1896 sera from day seven to day 70 post-infection which were used as a heterologous control (Figure 5.4b). In this instance, there was a progressive rise in ELISA values up to day 49 post-infection followed by a decline to day 70 post infection.

In contrast, the assay to determine the IgG response of the host to T. congolense TREU 1457 metacyclics showed similar patterns for all three experimental animals (Figure 5.5). The absorbance values rose sharply to day 21 post-infection although the response of rabbit 1 peaked at a higher level than the other two experimental animals. Although ELISA values fluctuated throughout the experimental period, particularly for rabbit 1, values for all three experimental animals never returned to pre-infection levels. The absorbance values obtained from antisera from rabbit 3 continued to rise from day seven to day 56 post-infection. Control ELISA values from antisera to a heterologous stock (TREU 1896) showed a slight but insignificant rise to 49 days post-infection.

5.4 DISCUSSION

The aim of the experiments described in this chapter was to produce an ELISA which could distinguish between different populations of metacyclic trypanosomes using NRS, homologous and heterologous antisera. The assays used intact in vitro-derived metacyclic

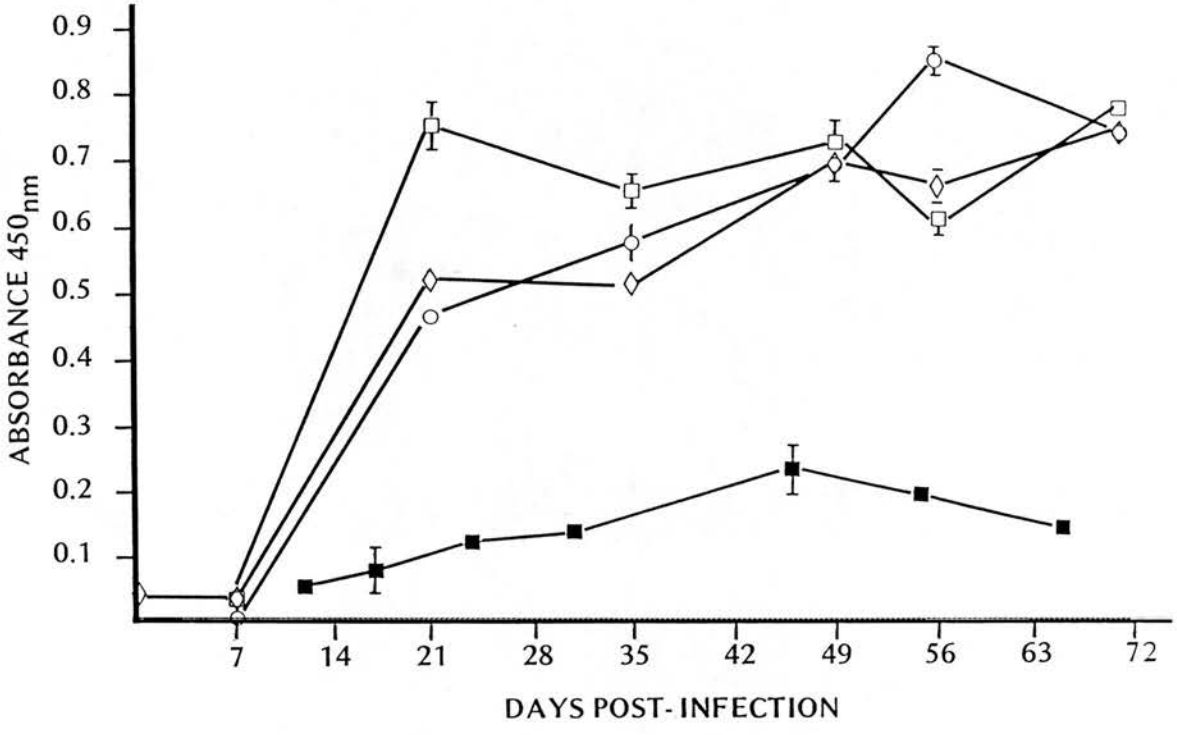


FIGURE 5.5

Examination by ELISA of the host's IgG response to TREU 1457 metacyclics using sera from three rabbits infected with TREU 1457 (□ ; ○ ; ◊) and a heterologous control from a rabbit infected with TREU 1896 (■).

forms of T. congolense and were adapted to examine the host's specific IgG and IgM response to the metacyclic population of one stock, T. congolense TREU 1457 over a period of 70 days post-infection.

The major difference between the ELISA technique described here and previous ELISAs involving trypanosomes was the preparation of the antigen: this was attained by using intact trypanosomes fixed to the wells of ELISA plates. The method developed ensured that sufficient numbers of trypanosomes were used, that they attached to the plastic and that they were adequately fixed.

10^6 in vitro-derived metacyclic trypanosomes per well produced the greatest differences in ELISA values between NRS, homologous and heterologous antisera. However, 10^4 metacyclics per well also produced considerable differences in ELISA values (Table 5.1). Insect form cultures produced between $3x10^5$ and $2x10^7$ metacyclics per flask depending on the stock used. More plates could be prepared from fewer culture flasks when 10^4 metacyclics per well were used to prepare each plate. Therefore, the number of metacyclic trypanosomes used to coat each well was 10^4.

Glutaraldehyde proved to be a better fixative for the metacyclics in this assay since it preserved the variant specific antigens of both TREU 1881 and TREU 1457 trypanosomes. Formalin fixation was successful with TREU 1457 metacyclics but not with TREU 1881 trypanosomes as demonstrated by the difference in absorbance values between homologous and heterologous antisera (Table 5.2). The optimal time for glutaraldehyde fixation of in vitro-derived metacyclics was ten minutes (Table 5.3).

Pre-treatment of wells of the ELISA plates by poly-L-lysine was used in an attempt to improve attachment of the metacyclics. It was shown that poly-L-lysine used on its own did not compare favourably with either glutaraldehyde fixation alone or poly-L-lysine pre-treatment followed by glutaraldehyde fixation (Table 5.4). Poly-L-lysine pre-treatment of plates prior to fixation has been recommended for some cell types particularly immunocytes (Heuser et al, 1981). It has also been shown that fixation with glutaraldehyde might be unnecessary, suggesting that poly-L-lysine was acting as a mild fixative as well as an attachment agent (Epstein and Lunney, 1985). However, those experiments used mammalian cells and it appears from the present experiments that intact metacyclic trypanosomes do require fixation before being used in the ELISA.

Glutaraldehyde reacts strongly with proteins and this often denatures protein antigens (Posner, Antoniou, Griffin, Schlossman and Lazarus, 1982). Non-specific binding to glutaraldehyde-fixed cells can also lead to an increase in background ELISA values (Lansdorp et al, 1980). Although these criticisms of glutaraldehyde fixation appear to be valid, certainly for some mammalian cells, there was no evidence of either antigen modification or of increased background when glutaraldehyde was used to fix metacyclic forms of T. congolense in the experiments described here.

Fixation of trypanosomes by glutaraldehyde and formalin has been compared directly using IFAT (Nantulya and Doyle, 1977). Optimal fixation by glutaraldehyde was found to be at 0.05% (v/v) for 60 seconds. At lower concentrations there was a loss of trypanosome

morphology whereas higher concentrations led to a loss of antigenicity. A fixation time of above 60 seconds was found to diminish specificity markedly. Formalin fixation produced good results consistently both in terms of preservation of trypanosome morphology and surface antigens. However, in the present experiments formalin fixation of attached trypanosomes compared unfavourably with glutaraldehyde. It is possible that formalin fixation requires cells to be in suspension to be effective. Jenni (1977a) reported that formalin fixation of trypanosomes in a blood smear was poor compared to fixation of bloodstream form trypanosomes which were in suspension. Successful glutaraldehyde fixation at 0.25% (v/v) has been achieved with trypanosomes in RIA, where the trypanosomes had previously been centrifuged onto the plastic wells of a microtitre plate (Morrison et al, 1982). This particular method is directly comparable to the technique used in these experiments with metacyclic trypanosomes.

An increase in serum IgM levels of infected rabbits was detectable by seven days post-infection (Figure 5.4). However, there was no distinct pattern through the next 70 days of the infection with each of three rabbits in the experimental group showing a different pattern of ELISA values. Antisera to a heterologous stock was used as a positive control and also showed a rise in serum IgM levels although the rise here was less marked and peaked at 49 days post-infection.

The ELISA values for NRS of two of the three rabbits from the homologous infection experiment group were between 0.2 and 0.3. This was higher than the background values obtained in previous ELISAs.

Such a result is not uncommon in IgM antibody assays because IgM is a more avid molecule than IgG and also there are likely to be differences in the immunoglobulin content of individual animals (Meurman, 1983). Non-specific binding of immunoglobulins can be decreased by preincubation of the ELISA plate with inactive proteins such as bovine serum albumin, casein or gelatin in order to block the non-specific binding sites (Kenna, Major and Williams, 1985; Vogt, Phillips, Henderson, Whitfield and Spierto, 1987). Foetal calf serum has also been used to block non-specific binding (Fairchild and Moorhead, 1985). Another approach is to dilute serum samples and conjugates with buffers containing Tween 20. This approach in conjunction with pre-incubating the plates with bovine serum albumin was followed in the ELISA reported here.

The production of non-trypanosome specific factors such as rheumatoid factor has been demonstrated in animals infected with trypanosomes (Klein, Mattern, Kornman and Bosch, 1970). Rheumatoid factor is an anti-IgG antibody of the IgM class and the way in which it could interfere with IgM assays is by attaching to the trypanosome specific IgG antibodies bound to the antigen in the solid phase and the anti-IgM conjugate subsequently binding to the rheumatoid factor (Voller and Bidwell, 1986). However, it is unlikely that this was involved here because the serum was diluted to 1:3200 and rheumatoid factor would have to have been present at a particularly high level.

Cattle infected with T. brucei or T. congolense produce high levels of neutralizing and phagocytosis promoting antibodies against the infecting as well as subsequent VATs that arise during the

infection (Musoke et al, 1981, Masake et al, 1983, Ngaira et al, 1983 and Nantulya et al, 1984). The detection of neutralizing antibodies to the infecting VATs 134 days post-infection from a cow infected with T. congolense suggests that these antibodies persist (Wilson and Cunningham, 1972) although it was thought that this was caused by a reappearance of the infecting VATs due to exhaustion of the antigenic repertoire. When single bloodstream form VATs had been used to initiate infections in cattle, recurring peaks of IgG and IgM to the infecting and subsequent VATs were noted and again thought to be caused by recurrent VATs (Musoke et al, 1981; Masake et al, 1983; Nantulya et al, 1979). In the experiments described here using the entire M-VAT repertoire and not just cloned populations expressing single VATs, recurrence of IgG peaks did not occur but neither did the ELISA values fall significantly having peaked at day 21 for the IgG response (Figure 5.5).

It is unlikely that the M-VATs are being expressed throughout the 70 day period but it is possible that antibodies against the M-VATs persist for some considerable time after infection. Persistence of antibodies was demonstrated by ELISA in rabbits infected with T. evansi (Luckins, Gray and Rae, 1978; Luckins, Boid, Rae, Mahmoud, El Malik and Gray, 1979). In these studies, ELISA values remained higher than pre-infection levels for up to 118 days post-treatment.

Although the IgM test showed considerable cross reactions between homologous and heterologous antisera, the assay designed to detect M-VAT specific IgG was shown to be serodeme specific throughout the seventy day infection period. ELISA values rose rapidly to 21

days post-infection and then stayed higher than pre-infection levels for the remainder of the infection. It appears, therefore, that this ELISA could be used to distinguish between different M-VAT repertoires in serological analyses.

CHAPTER SIX

THE DEVELOPMENT OF A VAT-SPECIFIC INDIRECT FLUORESCENCE ANTIBODY TEST USING *IN VITRO*-DERIVED METACYCLIC FORMS OF *TRYPANOSOMA CONGOLENSE*

6.1 INTRODUCTION

The indirect fluorescent antibody test (IFAT) is one of the most widely used field tests for the serodiagnosis of African trypanosomiasis. It is among the most sensitive of serodiagnostic tests, detecting levels of anti-parasite antibody as low at 1 µg per dl (Stites *et al*, 1982). However, the number and complexity of trypanosome antigens has resulted in poor specificity and problems in differentiating between trypanosome sub-genera (Dukes, Rickman, Killick-Kendrick, Kakoma, Wurapa, de Raadt and Morrow, 1984; Molyneux, 1975). As with other immunoassays, IFAT is dependent on the choice of antigen used for the test, the preparation of that antigen and the use of the test sera. Although it was claimed that the specificity of IFAT did not extend to antigenic variants of *T. brucei* or *T. congolense* (Wilson, Cunningham and Kimber, 1967), it is now known that IFAT not only detects common antigens (Wilson and Cunningham, 1971; Schindler, 1972) but that it is sufficiently sensitive and specific to differentiate between variable antigens (Van Meirvenne *et al*, 1975a).

The assay is based on the principle that specific antibody binds to the antigen that stimulated its production. The antigen-antibody complex is located using compounds which are fluorescent dyes or

fluorochromes. When fluorochromes are illuminated by light of a particular wavelength energy is released as light of a longer wavelength. The most commonly used fluorescent dyes or fluorochromes are fluorescein isothiocyanate (FITC) and tetramethyl rhodamine isothiocyanate (TRITC). They have characteristic absorption and emission spectra: FITC has an absorption maximum between 490 and 495 nm and it emits a characteristic green colour at 517 nm whereas TRITC emits red light and has an absorption maximum of 550 nm and a maximal emission of 580 nm (Goldman, 1968; Stites et al, 1982).

There are two distinct types of fluorescence assay: a direct method and an indirect method. In the direct method, the fluorochrome is conjugated directly to the primary antibody without altering its biological function (Coons, Creech and Jones, 1941), whereas in indirect tests the antigen is initially located with an unlabelled primary antibody and the antigen-antibody complex is subsequently recognized with fluorochrome conjugated anti-immunoglobulin. The indirect method has many advantages over the direct method. By double labelling, the gain in sensitivity of the indirect test over the direct test has been assessed to be ten-fold (Coons, 1956). Other advantages are that only a single fluorescent labelled antiglobulin is required for each host species, small amounts of sera can be tested and any loss of antibody titre due to conjugate preparation can be avoided (Coons, 1956; Goldman, 1968). Also, it is feasible to serially dilute serum samples until they are no longer recognized by the fluorescent antibody, thus providing a method of titrating antibody which cannot be achieved using the direct method (Bonniwell, 1975).

The first field evaluations of IFAT in bovine trypanosomiasis were made in Kenya (Wilson et al, 1967). Antigens were prepared by making blood smears from heavily infected blood followed by heat fixation. These early tests indicated that IFAT was useful for screening herds, perhaps in conjunction with conventional parasitological diagnostic methods but was unreliable on an individual animal basis. It was established that the sub-genera Trypanozoon, Nannomonas and Duttonella could be differentiated by end-point titres but attempts to differentiate members of the sub-genera Trypanozoon were unsuccessful (Wilson et al, 1967; Wilson, 1969). By separating trypanosomes from host blood components, air drying onto microscope slides and fixing the trypanosomes in 5% formalin it was possible to differentiate between Trypanozoon sub-species (Latif and Adam, 1973). This demonstrates the importance of antigen preparation in the results of the test since all other aspects of the assay were the same.

Responses to non-pathogenic species of trypanosome such as T. theileri produce cross reactions with pathogenic trypanosomes but appropriate dilutions of the test antisera can overcome this problem. Thus, Wilson et al, 1967 used dilutions of 1:40 as a baseline to compensate for cross reactivity when using heat-fixed bloodsmears. Zwart, Perie, Keppler and Goedbloed (1973) found it necessary to dilute sera to 1:160 to overcome the same problems when testing sera on acetone-fixed blood smears. Acetone-fixed trypanosomes fluoresce weakly in negative control sera making the differentiation between positive and negative sera difficult. The inherent subjectivity of

IFAT is a disadvantage of the assay because it is apparent that the results vary from one investigator to another.

Many different approaches to the preparation and fixation of trypanosomes for serodiagnostic IFAT have been used with varying degrees of success for diffferent assays. These include fixation with methanol (Weitz, 1963; Latif, 1972; Zwart et al, 1973), hydrochloric acid (Ashkar and Ochilo, 1972; Zwart et al, 1973; Latif, 1972) and acetic acid (Zwart et al, 1973). Other workers have reported that air-dried, unfixed trypanosomes could be used (Wery, Van Wettere, Wery-Paskoff, Van Meirvenne and Mesatewa, 1970a).

The possibility of using immunofluorescence as a technique to study antigenic variation within trypanosome populations was first investigated by Van Meirvenne et al (1975a).The trypanosomes used as antigens in the assay were mildly heat-fixed by drying them onto microscope slides at 37^{o}C for one hour. Sera were raised against cloned trypanosomes and the ability to identify specific variant antigens was achieved by adjusting the dilution of the clone specific sera and incorporating Evan's Blue as a counterstain to remove non-specific staining. At an appropriate dilution of antisera, the heterologous antigenic types appeared red coloured or matt green whereas trypanosomes expressing the homologous antigen showed brilliant green fluorescence.

The importance of both the choice of antigen and antisera used in IFAT was demonstrated by Jenni (1977a; 1977b) who used viable metacyclic and bloodstream forms of T. brucei in suspension to examine variant antigens. Although the use of viable cells can be laborious, the trypanosome surface antigens are in the form presented

to the host and therefore possible artefacts due to cell fixation can be avoided. However, on application of homologous antiserum at 37°C the surface variable antigen moves to the flagellar pocket region in a process known as capping (Barry, 1979). If capping occurs, the cell surface antigens cannot be observed by immunofluorescence. Capping did not occur in the IFAT demonstrated by Jenni (1977a, 1977b) who was able to identify serodemes by fluorescence. However, he concluded that within a given serodeme, the metacyclic population was homogeneous. The major fault in this assay was not in antigen selection or preparation but in the preparation of the antisera. Antisera to the metacyclic population was raised in rabbits infected by fly bite and the production of antibodies to all M-VATs present resulted in complete labelling of the population.

The need for monospecific sera against M-VATs was identified by Le Ray *et al* (1978). Using monospecific sera raised to cloned bloodstream form trypanosomes and cross reacting this sera with acetone-fixed bloodstream form and metacyclic trypanosomes these authors identified some but not all of the metacyclic population of one serodeme. Sera were later produced to a single M-VAT of *T. brucei* by isolating an antigenically stable clone from a rabbit at a time when its serum showed activity towards metacyclic trypanosomes (Barry, Hajduk and Vickerman, 1979). Monospecific antisera to this clone reacted with up to 20% of the metacyclic population indicating that the population was heterogeneous with respect to VAT. This approach was extended to show that at least four M-VATs were presented in one serodeme of *T. brucei* (Hajduk, Cameron, Barry and Vickerman, 1981). Monoclonal antibodies have since been used with IFAT to

confirm that the metacyclic population is heterogeneous with respect to VAT (Crowe et al, 1983; Nantulya et al, 1983; Prain and Ross, 1988).

The aim of the following experiments was to produce a reliable, sensitive and specific immunofluorescence assay with which it was possible to detect differences in M-VAT populations using fixed, in vitro-derived metacyclics of T. congolense. In order to achieve this, it was necessary to identify at least 90% of each homologous population. To eliminate false positive cross reactions, the best method of antigen preparation and optimal assay conditions were determined.

6.2 MATERIALS AND METHODS

6.2.1 Animals

(a) Mice

Female outbred mice (Tuck and Son) were used to infect tsetse flies, clone trypanosomes and to maintain trypanosomes prior to cryopreservation.

(b) Rabbits

Female NZW rabbits (Bantin and Kingman) were used to raise antisera for all the trypanosome stocks and to maintain tsetse flies for fly-derived infections.

(c) Tsetse flies

Glossina morsitans morsitans pupae were supplied by Dr. A.M. Jordan, Tsetse Research Laboratories, Bristol and were raised as

described in 3.6.2. Flies were infected with the uncloned trypanosome stocks and used to transmit infections to rabbits.

6.2.2 Trypanosomes

Stocks of T. congolense used as antigens and to infect laboratory animals are shown in Table 6.1. The histories of these stocks are given in Appendix 1.

Groups of three rabbits were used to raise antisera to each of the trypanosome stocks. Serum samples were collected from day 0 to day 56 post infection and stored in 1.8 ml cryopreservation tubes at -40°C until used.

6.2.3 Fixation of in vitro-derived metacyclic trypanosomes

6.2.3.1 Formalin fixation

Metacyclic trypanosomes were suspended in 9 ml of PSG pH 8.0, and one ml of a 10% formalin solution was then added. The trypanosomes were stored at 4°C overnight and then washed three times at 4°C in PBS pH 8.0, by centrifugation at 200 g. After washing they were resuspended in PBS pH 8.0 at a concentration of 10^7 metacyclics per ml. The metacyclics were added in 10 µl amounts to 15 well multitest slides (Flow Laboratories, UK), air dried and stored at -40°C in silica gel until used. The slides were brought to RT before being prepared for IFAT.

6.2.3.2 Acetone fixation

In vitro-derived metacyclic trypanosomes were prepared at a concentration of 10^7 trypanosomes per ml in PSG pH 8.0. The trypanosomes were added to 15 well multitest slides in volumes of 10 µl per well and the excess trypanosome suspension was immediately removed.

TABLE 6.1

Stocks of T. congolense used as antigens and to raise antisera in rabbits.

Antigen	Antisera
TREU 1457	TREU 1457
TREU 1627	TREU 1627
TREU 2037	TREU 2037
TREU 2034	TREU 2034
TREU 1894	TREU 1896
TREU 1881	TREU 1881
TREU 1885	TREU 1885
	TREU 1844
	TREU 1849

The slides were air dried and then immersed in acetone (BDH Chemicals Ltd) for ten minutes. After fixation, the slides were stored at -40°C in silica gel. Before use, the slides were brought to RT.

6.2.4 Examination of cross reactions obtained by IFAT

Metacyclic trypanosomes were formalin-fixed as described previously (6.2.3.1). All antisera were used at a dilution of 1:160 based on the results shown in Table 6.2. At this dilution, trypanosomes fluoresced with homologous antiserum but were negative with NRS and heterologous antiserum. Trypanosomes were counted using phase contrast and transmitted light. The number of fluorescing trypanosomes were then counted using incident light.

TABLE 6.2

Comparison of formalin and acetone fixation of metacyclic trypanosomes using two stocks of T. congolense TREU 1457 and TREU 1627 and reciprocal end point titres of homologous and heterologous antisera in IFAT.

		RECIPROCAL END-POINT TITRE OF ANTIBODY			
		ANTIGEN			
		Formalin fixation		Acetone fixation	
Antiserum	Days post-infection	TREU 1457	TREU 1627	TREU 1457	TREU 1627
TREU 1457	NRS	20	20	20	20
	14	ND	ND	ND	ND
	21	320	20	2,560	640
	30	ND	ND	ND	ND
	35	1,280	80	2,560	1,280
	56	2,560	320	10,240	1,280
TREU 1627	NRS	20	20	20	20
	7	20	40	20	40
	14	20	40	20	40
	21	ND	ND	ND	ND
	30	20	320	40	320
	35	ND	ND	ND	ND
	56	ND	ND	ND	ND

ND Not determined

6.2.5 Pre-incubation of formalin-fixed metacyclic trypanosomes with Concanavalin A

To determine whether the cross reactions observed with heterologous stocks were caused by exposed sugars on fixed trypanosomes or on trypanosomes with incomplete surface coats, the parasites were pre-incubated with the lectin Concanavalin A.

The optimal concentration of Concanavalin A to use in the assay was determined using Concanavalin A/FITC conjugate (Sigma Chemicals Ltd.) at a concentration of one mg per ml diluted serially using two-fold dilutions from 1:10 to 1:2560 in PBS pH 8.0. The conjugate

was then applied to the wells of 15 well multitest slides containing formalin-fixed metacyclic trypanosomes of TREU 1627 and TREU 1894. The slides were incubated for 30 minutes before being washed three times in PBS pH 8.0 and mounted for examination by fluorescence microscopy.

For the pre-incubation, Concanavalin A (Sigma Chemicals) was applied to each well of a 15 well multitest slide at a concentration of 250 µl per ml and the slides were incubated for 30 minutes. The slides were then washed three times in PBS pH 8.0 and then prepared for fluorescence microscopy.

6.2.6 The effects of the grade of formalin on metacyclic fixation

To examine whether the fixation of metacyclics was affected by the quality of the formalin used, two grades of formalin were used to fix the metacyclic trypanosomes of one stock of T. congolense, TREU 1881. Formaldehyde solution 37% (w/v), (BDH Chemicals Ltd., UK) containing 10-14% methanol was designated 'Normal' and Formaldehyde EM 36% (w/v), (TAAB Laboratories, UK) was designated 'EM grade'. Both solutions of formalin were used to fix the trypanosomes by the method described in 6.2.4.1.

Antisera to five stocks of T. congolense, TREU 1881, TREU 2034, TREU 1885, TREU 2037, and TREU 1896, were tested against these metacyclics.

6.2.7 Heat inactivation, fractionation and absorption of serum

Twenty one day post-infection serum from rabbit 1656 infected with in vitro-derived metacyclic forms of T. congolense TREU 2037 was used for the following three manipulations of sera.

6.2.7.1 Heat inactivation

The serum sample was diluted to 1:10 in PBS pH 8.0 and incubated at 65°C in a water bath for 30 minutes. It was then stored at -40°C until used.

6.2.7.2 Fractionation

To separate IgG class antibodies, an Affi-gel blue column (Bio-rad Laboratories) was packed to seven ml and cleaned with 2M NaCl. The column was then equilibrated with Tris/NaCl buffer at pH 8.0. One ml of a 1:10 dilution of serum was dialysed overnight in one litre of PBS pH 8.0. The dialysed serum was then passed through the column and collected in one ml fractions. Fractions four to twelve were pooled giving a total protein content of 6.5 mg. The protein was then precipitated in saturated ammonium sulphate which was added dropwise over a 30 minute period followed by centrifugation at 2000 **g** for 30 minutes at 4°C. The resulting pellet was resuspended in 0.8 ml of PBS pH 8.0 and dialysed overnight in one litre of PBS pH 8.0 at 4°C. The fraction was tested by IFAT using IgG and IgM specific FITC conjugates and found to be IgG. This IgG fraction of the antiserum from rabbit 1656 was then stored at -40°C until used.

6.2.7.3 Absorption of serum

A 1:10 dilution of the serum was added to intact, *in vitro*-derived insect forms, including metacyclics of *T. congolense* TREU 1676, a known heterologous stock isolated from Tanzania. The trypanosomes and serum were incubated at 37°C for one hour and then centrifuged at 200 **g** for 15 minutes. The process was then repeated twice before storing the absorbed serum at -40°C until used.

6.2.8 General preparation of fixed antigen smears for immunofluorescence

Fixed metacyclic trypanosomes on 15 well multitest slides were incubated with antisera at various dilutions in a humid chamber at RT for 15 minutes. The slides were then washed three times in PBS pH 8.0 and allowed to stand in the final wash for ten minutes. The area around each test well was dried and the GAR/FITC conjugate (Nordic Immunologicals, UK) was added at a dilution of 1:100, with Evan's Blue incorporated at a dilution of 1:10,000 acting as a counterstain. The slides were incubated again in the humid chamber for 15 minutes and washed three times in PBS pH 8.0 as before. After the final wash the slides were dried and mounted in 60% PBS/glycerol before being examined by fluorescence microscopy using the technique described in Chapter Four.

6.2.9 Fluorescence assay using living trypanosomes

Immunofluorescence using viable *in vitro*-derived metacyclic forms of TREU 1881 and TREU 1627 was carried out in 96 well microtitre plates at $4^{o}C$. 10^{6} metacyclics in PSG pH 8.0 were added to each well and antisera at dilutions of 1:4, 1:8, 1:16, 1:32 and 1:64 were then added. The plates were incubated at $4^{o}C$ for 30 minutes. After incubation, the plates were centrifuged at 200 **g** for 15 minutes, the sera removed and the trypanosomes resuspended in PSG pH 8.0 and stored for a further 15 minutes at $4^{o}C$. This washing procedure was repeated three times. After the final wash, the trypanosomes were resuspended in GAR/FITC at a dilution of 1:20, 1:40 or 1:80 containing a 0.05 mg per ml solution of propidium iodide and incubated for 30 minutes. The trypanosomes were washed and centrifuged as before

then resuspended in PSG pH 8.0 and transferred to microscope slides and examined.

6.2.10 Immunofluorescence by the triple labelling method

The method of preparing the antigen smears for fluorescence was essentially the same as was described previously (6.2.8) except for the addition of a GAR/IgG second antibody. Formalin-fixed metacyclic trypanosomes were incubated with antisera for 15 minutes and then washed. After washing, GAR/IgG (Nordic Immunologicals) was added to each well at a dilution of 1:50, 1:100, 1:150, 1:200 or 1:300 and the slides were incubated for a further 15 minutes. After washing, RAG/Ig/FITC (Nordic Immunologicals) was added to each well at a dilution of 1:100. The slides were then washed a further three times before being mounted for examination by fluorescence microscopy.

6.3 RESULTS

6.3.1 Comparison of acetone and formalin fixation of metacyclic forms of T. congolense as determined by IFAT

Acetone and formalin were used to fix in vitro-derived metacyclic trypanosomes of TREU 1627 and TREU 1457 for use in IFAT and the results are shown in Table 6.2. The end-point titre for NRS was 1:20 for both methods of fixation on both stocks of trypanosomes and therefore the fixation had no effect on the results for sera from uninfected animals. Acetone-fixed metacyclics showed higher end-point titres of homologous antiserum than formalin-fixed trypano-

somes. For example, 21 day post-infection antiserum had a titre of 1:2560 when the trypanosomes were acetone-fixed compared to an end-point titre of 1:320 when formalin-fixed trypanosomes were used. However, acetone-fixed TREU 1627 metacyclics also gave considerably high end-point titres for the heterologous reaction with anti-TREU 1457 serum; 1:650 using day 21 post-infection serum. Although formalin fixation of TREU 1457 and TREU 1627 metacyclics produced lower end-point titres than acetone-fixed trypanosomes with the homologous antisera, the corresponding reactions with heterologous antisera were also reduced markedly.

6.3.2 Examination of cross reactions obtained by IFAT between different stocks of T. congolense

The relationships between stocks of T. congolense were examined by IFAT using as antigens in vitro-derived metacyclic trypanosome populations of five stocks isolated in Zambia and one stock isolated in The Gambia. The results of these assays are shown in Table 6.3 and are based on the intensity of fluorescence, classed either as being positive, as shown in Figure 6.1 or negative, as shown in Figure 6.2 with the proportion of positively fluorescing trypanosomes expressed as a percentage. With homologous antigen/ antibody reactions, the results show that all the animals in each group gave a positive response recognising at least 90% of the total metacyclic population and in most cases the entire metacyclic population was recognized by 21 day post-infection homologous antiserum. There were however, cross reactions between stocks (Figure 6.3); for example, three animals infected with TREU 1881 reacted with 21-26% of TREU 1894 metacyclics. Also, at least one animal in each of the

TABLE 6.3

Examination by IFAT of cross reactions obtained between different stocks of T. congolense using in vitro-derived metacyclic forms as antigens and 21 days post-infection rabbit antiserum.

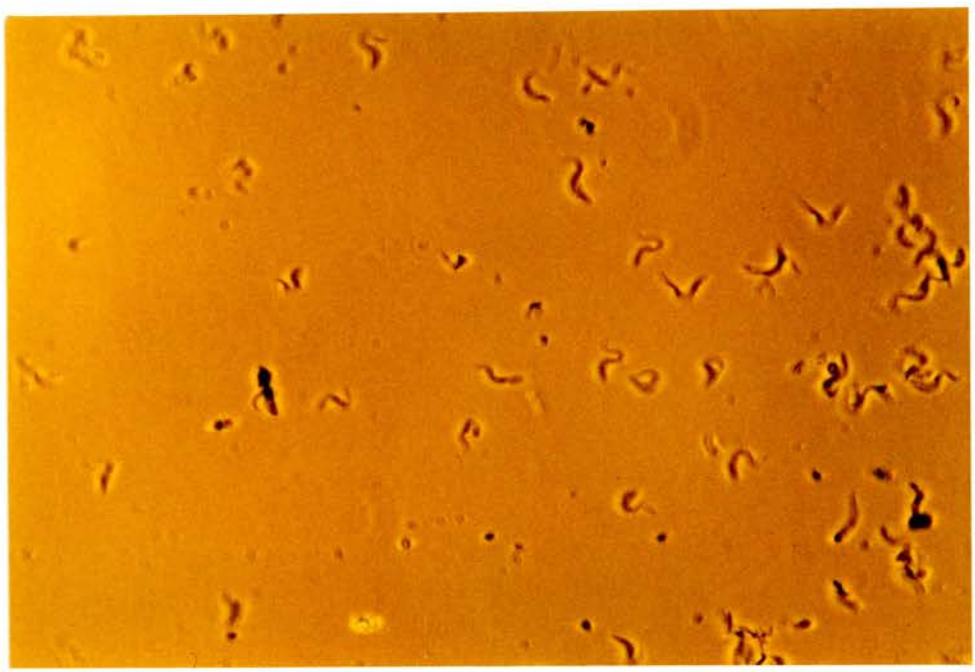
		Proportion of fluorescing trypanosomes					
		ANTIGEN					
Antiserum (1:160)	Rabbit number	TREU 1627 %	TREU 2037 %	TREU 2034 %	TREU 1894 %	TREU 1881 %	TREU 1885 %
TREU 1881	1645	-	-	-	26	**94**	-
	1661	26	-	-	26	**98**	-
	1662	-	-	-	22	**100**	-
TREU 1884	1695	-	-	-	-	-	-
	1692	45	-	13	12	-	5
	1693	54	-	9	23	-	22
TREU 1849	1597	16	-	12	18	14	8
	1598	-	-	-	-	-	-
	1603	-	-	-	-	-	-
TREU 1885	1667	38	-	-	12	20	**92**
	1687	-	-	-	-	-	**90**
	1682	-	-	-	-	-	**100**
TREU 2037	1660	-	**100**	-	-	-	-
	1657	20	**100**	-	-	-	-
	1656	15	**100**	-	11	-	16
TREU 2034	1566	-	-	**100**	-	-	-
	1563	50	-	**100**	-	-	9
	1562	-	-	**100**	-	-	-
TREU 2034	1614	-	-	**100**	-	-	-
	1610	14	-	**100**	-	-	-
	1680	-	-	**100**	-	-	-

+ positive fluroescence - negative

FIGURE 6.1

Formalin-fixed *T. congolense* TREU 2037 metacyclics labelled with 21 day post-infection anti-TREU 2037 rabbit serum and GAR/FITC (incorporating Evan's Blue). Almost 100% of the trypanosomes observed using phase contrast are fluorescing when viewed under ultra-violet illumination.

(x 425 magnification)



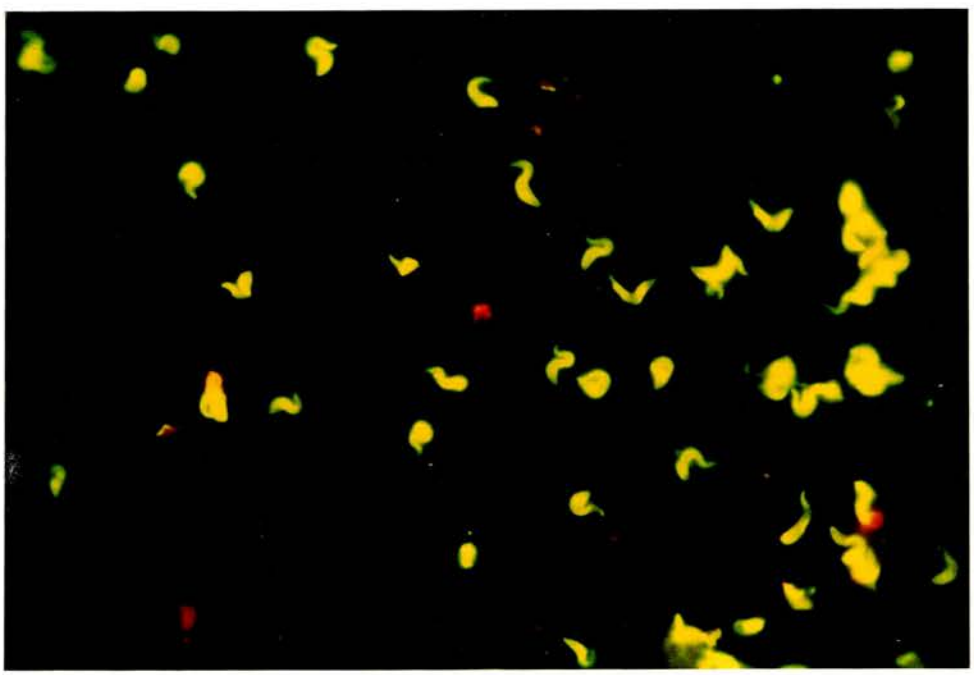
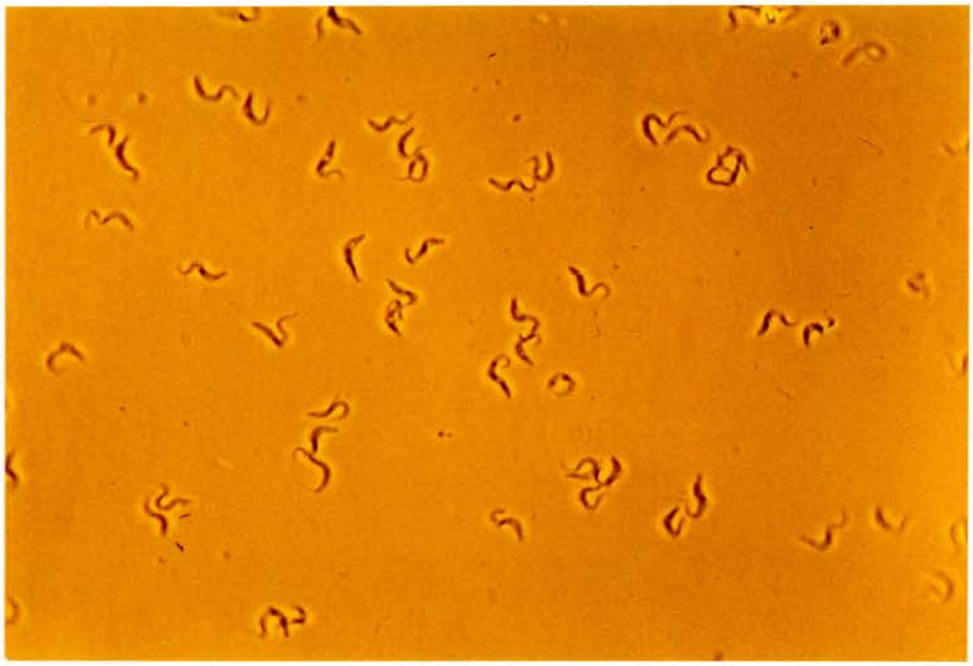


FIGURE 6.2

Formalin-fixed *T. congolense* TREU 2037 metacyclics labelled with 21 day post-infection anti-TREU 1881 rabbit serum and GAR/FITC (incorporating Evan's Blue). All the trypanosomes appear red under ultra-violet illumination indicating a negative reaction.

(x 425 magnification)



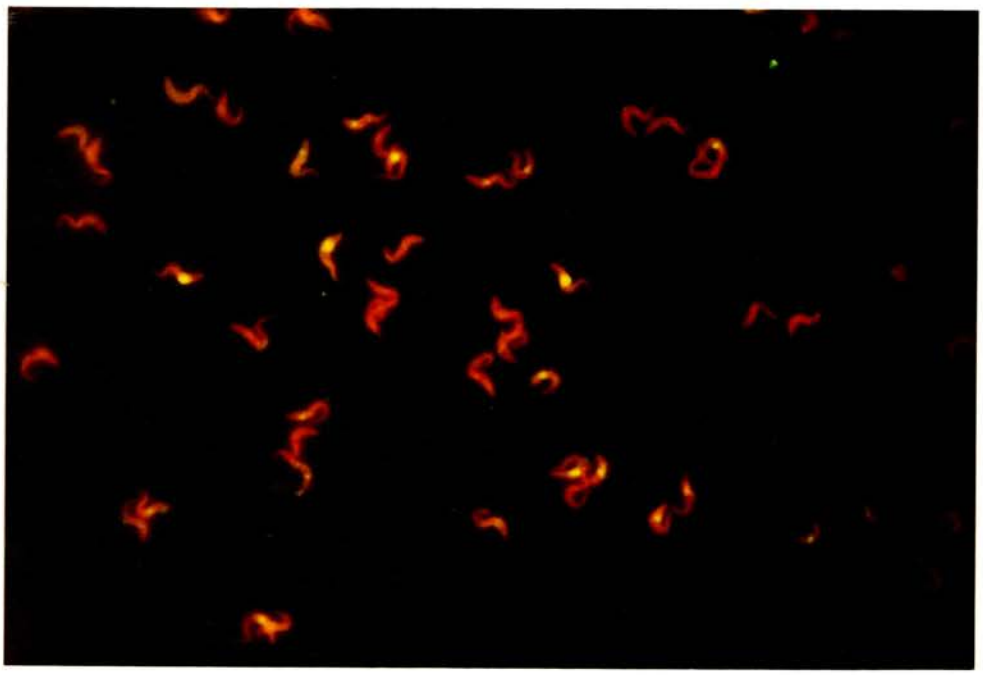
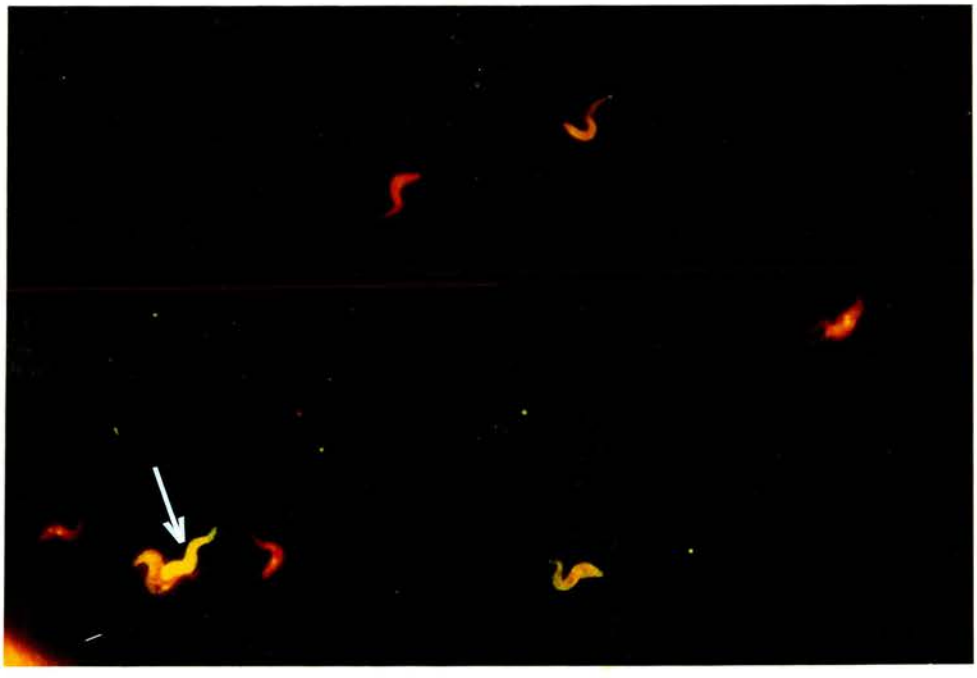


FIGURE 6.3

Formalin-fixed *T. congolense* TREU 1894 metacyclics labelled with 21 day post-infection anti-TREU 1881 rabbit serum and GAR/FITC (incorporating Evan's Blue). Single trypanosome (⟶) showing surface fluorescence under ultra-violet illumination. (x 425 magnification)



groups infected with one of the six Zambian stocks showed a positive reaction ranging from 12% to 54% with metacyclics of TREU 1627 isolated from the Gambia.

6.3.3 Pre-incubation of formalin-fixed metacyclics with Concanavalin A

To determine whether the cross reactions observed in Table 6.3 could be due to the effects of exposed sugars on poorly-fixed trypanosomes, metacyclics of two stocks TREU 1894 and TREU 1627 were pre-incubated with the lectin Concanavalin A. The optimal working concentration of Concanavalin A was determined using Concanavalin A/FITC conjugate and the results of this assay are shown in Table 6.4. Positive fluorescence, indicating lectin binding to some exposed sugars, was observed at a dilution of 1:80 (12.5 µg per ml) in one stock, TREU 1894. However, this binding was observed on the entire metacyclic population indicating that the cross reactions were not caused by exposed regions of the surface coats of a proportion of the metacyclic population. No lectin binding was observed on the formalin-fixed TREU 1627 metacyclics.

The assay was modified by pre-incubating formalin-fixed metacyclics of TREU 1894 and TREU 1627 with 250 µl per ml Concanavalin A before they were used in a fluorescence assay with antisera for stocks which had previously shown cross reactions (6.3.2). The results of this assay are shown in Table 6.5. No differences were observed between trypanosomes pre-incubated with the lectin and those prepared in the usual manner.

TABLE 6.4

Results of a fluorescence assay using formalin-fixed metacyclic trypanosomes of two stocks of T. congolense, TREU 1627 and TREU 1894 with Concanavalin A/FITC.

	RECIPROCAL END-POINT TITRES OF ConA/FITC (1 mg/ml)								
Antigen	10	30	40	80	160	320	640	1280	2560
TREU 1627	-	-	-	-	-	-	-	-	-
TREU 1894	+	+	+	+	-	-	-	-	-

+ positive fluorescence
- negative

TABLE 6.5

Results of the IFAT using TREU 1627 and TREU 1894 formalin-fixed metacyclics with or without pre-incubation of the trypanosomes with Concanavalin A (ConA).

		ANTIGEN			
		TREU 1627		TREU 1894	
		Pre-incubation		Pre-incubation	
Antiserum (1:160)	Rabbit number	with ConA	Without ConA	with ConA	Without ConA
TREU 1627	1514	+	+	-	-
TREU 2037	1660	-	-	-	-
TREU 2037	1656	-	+	+	+
TREU 1885	1682	+	-	-	-
TREU 1885	1667	+	+	-	-
TREU 1881	1645	-	-	+	+
TREU 1881	1661	+	+	+	+
TREU 2034	1614	+	-	-	-
TREU 2034	1610	+	+	-	-
TREU 1844	1695	+	-	-	-
TREU 1844	1692	+	+	+	+
TREU 1849	1598	-	-	-	-
TREU 1849	1597	+	+	+	-

6.3.4 The effects of the grade of formalin used for fixation of metacyclic trypanosomes on the IFAT

EM grade formalin used in preparing cells for electron microscopy was compared with standard formalin in its ability to preserve the metacyclic surface antigens of TREU 1881 for use in IFAT. The results of this comparative assay are shown in Table 6.6. Cross reactions were observed with antisera from two animals infected with TREU 1885 using both grades of formalin. Both methods of metacyclic fixation produced the same results in the subsequent IFAT.

6.3.5 The effects of heat inactivation, serum fractionation and absorption with other in vitro-derived culture forms on the cross reactions observed by IFAT

To determine whether a factor present in anti-trypanosome rabbit serum was causing the cross reactions observed in the IFAT, one 21 day post-infection serum sample from rabbit 1656 infected with TREU 2037 which consistently cross reacted with known heterologous stocks was treated in several ways to reduce this reactivity. The results are shown in Table 6.7.

When the untreated antiserum was used in IFAT with formalin-fixed metacyclics of TREU 1627, positive fluorescence was observed in 16% of the heterologous trypanosomes at an end-point titre of 1:320. This compared to 100% fluorescence of the homologous metacyclic trypanosomes at an end-point titre of 1:640. Similar end-point titres were observed when the antiserum had been heat inactivated at 65^{o}C for 30 minutes. Absorption of the serum with insect forms of TREU 1676, an unrelated stock isolated in Tanzania lowered the end-point titres of both the homologous and heterologous reactions and the difference in end-point titres were increased from one-fold

TABLE 6.6

The effects of the grade of formalin used for fixation of *T. congolense* TREU 1881 *in vitro*-derived metacyclics on results obtained in the immunofluorescence assay.

Antiserum (1:160)	Rabbit number	GRADE OF FORMALIN	
		Normal	EM
TREU 1881	1662	+	+
	1661	+	+
	1645	+	+
TREU 2034	1610	-	-
	1614	-	-
	1680	-	-
TREU 2034	1562	-	-
	1566	-	-
	1563	-	-
TREU 1885	1667	+	+
	1687	-	-
	1682	-	-
TREU 2037	1657	-	-
	1656	-	-
	1660	-	-
TREU 1896	1481	-	-
	1490	-	-
	1489	-	-

+ positive fluorescence
- negative
NRS all negative

TABLE 6.7

Reciprocal end-point titres obtained in IFAT after heat-inactivation, serum fractionation and absorption with other insect forms for an anti-TREU 2037 serum sample.

Antiserum	Treatment	ANTIGEN	
		TREU 2037	TREU 1627
TREU 2037	None	640	320
	IgG fractionation	160	40
	Heat inactivation	640	320
	Absorption	80	20
TREU 1627	None	<20	320

to two-fold. A third method of reducing cross reactivity of the serum sample was by using ion-exchange chromatography. In assays using IgG class antibodies the end-points were reduced to 1:160 for the homologous reaction and 1:40 for the heterologous reaction. Again, this difference in end-point titre was greater than that obtained using the untreated antiserum. Hence, non-specific cross reactions in IFAT can be eliminated by either absorption of the serum with other insect form trypanosomes or by fractionating the antiserum.

6.3.6 The use of viable metacyclic trypanosomes in IFAT

The effect of using unfixed trypanosomes on the results of the fluorescence assay was examined using viable TREU 1881 and TREU 1627 *in vitro*-derived metacyclic forms. Firstly, the optimal working dilutions of the sera and conjugate used in the assay were determined and the results of these preliminary assays are shown in Table 6.8. Control NRS did not react with the trypanosomes at any of the three

TABLE 6.8

Results of a fluorescence assay to determine optimal serum and conjugate dilutions using viable metacyclic trypanosomes of T. congolense TREU 1881, NRS, and a 21 day post-infection homologous antiserum.

Conjugate dilution	Serum	SERUM DILUTION 1:4	1:8	1:16	1:32	1:64
1:20	NRS	-	-	-	-	-
	21d pi	+	+	+	-	-
1:40	NRS	-	-	-	-	-
	21d pi	+	+	+	+<100%	-
1:80	NRS	-	-	-	-	-
	21d pi	-	-	-	-	-

conjugate dilutions or the five serum dilutions. The 21 day post-infection homologous antiserum reacted positively with the metacyclic trypanosomes when the FITC conjugate was at dilutions of 1:20 and 1:40 but not when it was used at 1:80. Positive fluorescence of 100% of the metacyclic trypanosomes was obtained at serum dilutions of 1:4, 1:8 and 1:16. Less than 100% positive fluorescence was obtained at a serum dilution of 1:32 when the conjugate was used at a dilution of 1:40. Having established that a serum dilution of 1:16 and a conjugate dilution of 1:40 were the optimal conditions for this assay, another series of tests were carried out using antisera to stocks of T. congolense which had cross reacted in previous assays with formalin-fixed trypanosomes. Table 6.9 shows that the only positive reactions observed occurred when using homologous antisera and metacyclic trypanosomes: there were no cross reactions between heterologous stocks.

TABLE 6.9

Results of an IFAT using viable metacyclic trypanosomes of TREU 1881, TREU 1627 and 21 day post-infection antisera which had previously cross reacted in assays using formalin-fixed trypanosomes.

Antiserum (1:160)	Rabbit number	ANTIGEN	
		TREU 1881	TREU 1627
TREU 1627	1514	-	+
TREU 2037	1657	-	-
TREU 2037	1656	-	-
TREU 1881	1661	+	-
TREU 1881	1662	+	-
TREU 1885	1667	-	-
TREU 1885	1687	-	-
TREU 2034	1563	-	-
TREU 1844	1693	-	-
TREU 1849	1597	-	-

+ positive fluorescence
- negative

6.3.7 The use of a triple label method in IFAT

The optimal working dilution of the GAR/IgG second antibody was determined first and the results of this assay are shown in Table 6.10. The antiserum was used at a dilution of 1:160 and the RAG/Ig/FITC conjugate was used at a dilution of 1:100. At any of these dilutions the NRS control did not react with either TREU 1881 or TREU 2037 metacyclic forms. The homologous reaction between TREU 1881 metacyclics and anti-TREU 1881 rabbit serum was positive when second antibody was diluted to 1:50 to 1:150 but negative at higher dilutions. The heterologous reactions using anti-TREU 2037 rabbit serum were negative at all the GAR/IgG dilutions. Correspondingly, the homologous reactions between TREU 2037 metacyclics and anti-TREU 2037 rabbit serum were positive at second antibody dilutions from 1:50 to 1:300 whilst the heterologous reactions using anti-TREU 1881 rabbit serum were all negative. Therefore, of the optimal working conditions for the triple label IFAT namely, a serum dilution of 1:160, GAR/IgG at a dilution of 1:150 and RAG/FITC conjugate at 1:100, the assay was used to examine antisera which had cross reacted with heterologous trypanosomes in previous assays (Table 6.11). No cross reactions were observed when the triple labelling method was used.

TABLE 6.10

Determination of optimal conditions for IFAT using rabbit antiserum, goat anti-rabbit IgG (GAR/IgG) and rabbit anti-goat immunoglobulin FITC conjugate (RAG/Ig/FITC).

Antigen	GAR/IgG dilution	ANTISERUM (1:160) NRS	21 day post-infection anti-TREU 1881	21 day post-infection anti-TREU 2037
TREU 1881	1:50	-	+	-
	1:100	-	+	-
	1:150	-	+	-
	1:200	-	-	-
	1:300	-	-	-
TREU 2037	1:50	-	-	+
	1:100	-	-	+
	1:150	-	-	+
	1:200	-	-	+
	1:300	-	-	+

+ positive fluorescence
- negative
RAG/Ig/FITC conjugate used at a dilution of 1:100

TABLE 6.11

Results of a fluroescence assay using the triple labelling method with antisera which had previously cross reacted with heterologous trypanosomes.

Antiserum (1:160)	ANTIGEN TREU 1627	TREU 1881	TREU 1885
TREU 1627	+	+	+
TREU 1881	-	+	-
TREU 1844	-	-	-
TREU 1885	-	-	+
TREU 2037	-	-	-
TREU 2034	-	-	-

+ positive fluorescence
- negative

6.4 DISCUSSION

The subjectivity of the IFAT has often been cited as a disadvantage of the test (Molyneux, 1975; Latif and Adam, 1973; Voller, 1977). It is the result of grading fluorescence from one plus (+) to three or four plus (+++ or ++++). Wery-Paskoff and Van Wettere (1970)*, regarded only bright (+++) or very bright (++++) fluorescence as positive. However, Wilson and Cunningham (1971), defined positive serum as one that gave a two plus (++) or brighter fluorescence at a dilution of 1:40.

In the assays described here, positive fluorescence is only recognized when trypanosomes are fluorescing green and negative fluorescence as when parasites are stained red with Evan's blue counterstain. Hence, by not defining degrees of fluorescence and using single serum dilutions the assays used here become less subjective than those reported previously.

Two methods of fixation, acetone and formalin, were compared and although higher end-point titres were observed in the homologous reactions using acetone-fixed than formalin-fixed metacyclics, the corresponding end-point titres for the heterologous reactions were also higher (Table 6.2). In general, the differences between the end-point titres of homologous and heterologous antisera were four serial two-fold dilutions when the trypanosomes had been fixed in formalin whereas when acetone-fixed trypanosomes were used those differences could be as low as one dilution factor.

Acetone is often used as the fixative of choice for trypanosomes in fluorescence assays to detect variant specific antigens (Barry et al, 1979; Magnus, Vervoort and Van Meirvenne, 1982) and to detect

* Wery, Wery-Paskoff and Van Wettere (1970a)

common antigens for serodiagnosis (Molyneux, 1975). As a fixative, acetone can allow penetration of immunoglobulins into mammalian cell cytoplasm (Biberfeld, Biberfeld, Molnar and Fagraeus, 1974) and also leads to an increase in background fluorescence in assays with trypanosomes due to exposed common antigens (Zwart et al, 1973). However, previous work has shown that by titrating the sera to a suitable end-point, the stronger immune response to the variant specific antigens allows VAT specific labelling at high antibody dilutions (Le Ray et al, 1978; Barry et al, 1979 and Hajduk et al, 1981).

Fixation of cells by formalin has been used successfully to stabilize surface membrane antigens of mammalian tumour cells (Drake, Ungaro and Mardiney, 1972 and Kudo, Aoki and Morrison, 1974) and bloodstream form trypanosomes (Nantulya and Doyle, 1977) where the parasites retained both their morphology and antigenicity. Fixation of the metacyclics by a one percent formalin solution at $4^{o}C$ overnight generally resulted in an assay which was VAT-specific (Tables 6.2 and 6.3). However, false positive cross reactions of between 9% and 54% of heterologous populations occurred in some cases. Serologically similar VATs, called iso-VATs, which cross react in immunolysis, agglutination and immunofluorescence are known to be present in different serodemes and different species of trypanosomes (Gray and Luckins, 1976; Van Meirvenne et al, 1975b; 1977; Magnus et al, 1982). The possibility that the assays described here were detecting iso-VATs was discounted because of the inconsistent patterns observed between stocks which were known to be antigenically distinct. Also,

no cross reactions were observed when viable trypanosomes were used in the assay.

It is possible that impurities in standard formalin, for example 10%-14% methanol, could adversely affect the fixation (Table 6.6). However, since there were no differences observed between the two grades of formalin, it was unlikely that cross reactions were the result of impurities in the fixative.

Preparation of the metacyclics may have resulted in damage to the parasites, possibly resulting in incomplete surface coats and this may have led to non-specific cross reactions. Metacyclics were pre-incubated with Concanavalin A (Table 6.5). Concanavalin A binds to the sugars α-D-mannose and α-D-glucose. Previous work has shown that these sugars are present on the cell surface of some stocks of T. congolense both in the bloodstream form (Jackson, Honigberg and Holt, 1978; Rautenberg, Reinwald and Risse, 1980 and Rovis, Barbet and Williams, 1978) and on the uncoated procyclic or insect midgut form (Mutharia and Pearson, 1987). It has been suggested that these sugars could be involved in eliciting an immune response of the host (Jackson et al, 1978). However, although all the metacyclic forms of one stock of T. congolense, TREU 1894 bound Concanavalin A, the cross reactions were not diminished by pre-incubating the formalin-fixed metacyclics with this lectin. Therefore, exposed sugars on poorly fixed or partially coated trypanosomes were not the cause of cross reactions.

The possibility that the heterologous cross reactions could be due to non-specific factors in trypanosome-infected rabbit serum was investigated by treating an antiserum in three ways: absorption with

insect forms of a heterologous stock; serum fractionation and heat inactivation (Table 6.7). Absorption of test serum with heterologous antigens is a technique frequently used to effect the removal of unwanted cross reactions (Goldman, 1968; Goddeeris, Katende, Irvin and Chumo, 1982; Katende, Musoke, Nantulya and Goddeeris, 1987). In this assay, although absorbed serum showed lower end-point titres for both the homologous and heterologous stocks, the difference in end-point titres between homologous and heterologous reactions increased compared to those using unabsorbed serum. Heat inactivation at 65^{o}C for 30 minutes is a crude but efficient method of removing IgM and complement components from serum (McKenzie and Boreham, 1974). However, when this treatment of serum was used here, there was no effect on end-point titres of the serum with either homologous or heterologous antigens. Fractionating was successful in removing cross reacting components from the serum. It would appear therefore that non-specific IgM or other non-specific serum factors were playing a role in causing cross reactions in these assays.

Immunofluorescence on viable, unfixed trypanosomes (Tables 6.8 and 6.9) was shown to be sensitive and specific and no heterologous cross reactions were observed. Viable trypanosomes have been used for immunofluorescence assays before (Jenni, 1977a, b) and the phenomenon of capping involving a redistribution of cell surface antigens when unfixed cells are bound by antibody (Taylor, Duffus, Raff and de Petris, 1971; Barry, 1979) did not occur in these experiments because here, the cells were maintained at 4^{o}C throughout the assay. The use of propidium iodide (Yeh, Hsi and Faulk, 1981 and Ockleford, Hsi, Wakely, Badley, Whyte and Faulk, 1981) was important since it enabled

the viability of the metacyclic trypanosomes to be assessed instantly. Propidium iodide does not penetrate living cell membranes and has an excitation spectrum of 400 to 540 nm and an emission spectrum of between 560 and 620 nm. Therefore propidium iodide emits red light in the same excitation spectrum that FITC emits green light allowing the two fluorochromes to be used together. However, the disadvantages of harvesting large numbers of trypanosomes, resuspension and centrifugation of cells between washing and staining precludes this test for the large scale screening of sera.

Although the cross reactions between heterologous stocks and sera were eliminated by fractionating serum a simpler technique was developed using an anti-rabbit IgG specific second antibody to identify the IgG component of whole rabbit serum (Tables 6.10 and 6.11). This assay was VAT-specific and involved few manipulations of trypanosomes or antisera when preparing the test.

Four techniques, absorption with heterologous antigens, fractionation of serum, use of living metacyclics and the triple labelling method produced fluorescence assays which were both sensitive and VAT-specific. Of these techniques, triple labelling was by far the least cumbersome and most applicable to examining large numbers of sera. This method was therefore used in later experiments to examine further the serological relationships of different trypanosome stocks isolated from East, West and Central Africa.

CHAPTER SEVEN

CHARACTERIZATION OF *TRYPANOSOMA CONGOLENSE* SERODEMES IN STOCKS ISOLATED FROM CHIPATA DISTRICT, ZAMBIA

7.1 INTRODUCTION

There is evidence to suggest that some *Bos indicus* breeds of cattle in East Africa may acquire a degree of resistance to trypanosome infection when they are maintained under strictly controlled drug regimes(Whiteside, 1962; Wilson *et al*, 1976). In Kenya, Wilson *et al* (1976) introduced and maintained a herd of breeding cattle in an area of medium trypanosome challenge. Chemotherapy using the short acting curative drug Berenil was given to animals on an individual basis whenever their packed cell volume (PCV) fell to below 20% or if they were clinically ill. Initially, the period between drug treatments was 50 days but this increased to 130 days by the ninth treatment and some steers which received no therapy for the last six months of the experiment continued to survive and grow at a similar rate to those receiving therapy. Control animals which received no therapy and were introduced at intervals during this experiment developed signs of severe trypanosomiasis and died. These observations suggest the presence in the study area of a limited number of serodemes and stability of those serodemes over a period of time.

The specificity of the acquired resistance, dependent on geographical distribution, stability and level of challenge

unfortunately means that its extent is limited. The immunity developed in one geographical area may not be effective when animals are moved to another area. In addition, acquired immunity to specific serodemes may not occur if the animals are exposed to a high trypanosome challenge of large numbers of serodemes which are constantly changing (Wilson, Le Roux, Paris, Davidson and Gray, 1975). Also, when the trypanosome challenge is too low to enable constant challenge of the same serodemes, immunity might fail to develop (Paling, Leak, Katende, Kamunya and Moloo, 1987). A better understanding of trypanosomiasis under field conditions including the distribution of serodemes would provide information essential for research aimed at developing improved control techniques and better serodiagnosis of infection.

Relatively little is known about antigenic variation in T. vivax and T. congolense compared to T. brucei because of the inability of many isolates of these parasites to establish infections in laboratory animals and difficulties in obtaining sufficient numbers of trypanosomes for serological assays. However, it is generally believed that observations on the T. brucei subgroup are applicable to the other pathogenic tsetse-transmitted trypanosomes. The capacity of trypanosomes for antigenic variation has been recognised for many years as a factor of considerable importance in practical and experimental studies on trypanosomiasis (Gray, 1969). Antigenic variation also warrants consideration as a limiting factor in attempts to use serological methods to classify strains of trypanosomes, to detect infected animals and to determine the species of infecting organisms.

The early work of Broom and Brown (1940) had demonstrated that passage through the tsetse fly caused relapse populations of a T. brucei strain to revert to a relatively stable 'parent type'. This work was extended to show that when a strain of T. brucei was passaged through flies, a common antigen or 'basic strain' antigen occurred in all its substrains isolated during the first seven days of infections in animals infected by different flies (Gray, 1965a). These findings were important for two reasons: serological typing was possible for trypanosomes using the metacyclic stage and if the number of basic strain antigens was limited then immunization would be possible.

Evidence that the metacyclic population was heterogeneous with respect to VAT emerged when T. brucei M-VAT specific antisera was used to examine infected tsetse salivary probes (Barry et al, 1979; Hajduk et al, 1981). However, it was discovered that although the population was heterogeneous for any given serodeme the same antigenically mixed population was always produced after each complete life cycle. Thus, the principle of basic strain antigenicity remained. While the majority of M-VATs are stable and serodeme specific, Barry, et al., (1983) showed that over several complete life cycles the individual M-VATs could be deleted or replaced.

Various methods have been employed to analyse the variable antigens present in metacyclic populations of T. congolense. Tsetse flies infected with T. congolense produce few metacyclics, therefore, early attempts to serologically type this species involved raising "primary parasitaemic populations" (Dar, Wilson, Goedbloed, Ligthart and Minter, 1973; Wilson, Dar and Paris, 1983). The neutralization

of infectivity test was used to compare trypanosome stocks which were isolated from four distinct geographical areas; Lugala (Uganda) and Kiboko, Masai-Mara, and Sindo (Kenya). Inter- and intra-area cross reactions were observed and within Lugala the number of antigenic variants was found to be large. The conclusion from this survey (Wilson et al, 1973) should be treated with caution for two reasons. Firstly, it is possible that antigenically, primary parasitaemic trypanosomes may not be related to metacyclics, particularly with T. congolense where long prepatent periods lead to the expression of new VATs before sufficient trypanosomes have been isolated. Secondly, most of the stocks isolated had a low infectivity for mice, making the neutralization of infectivity test unsuitable for this survey.

Another method used to analyse antigenic variants of T. congolense involved immunosuppressing laboratory animals by X-irradiation followed by feeding with infected tsetse flies has been attempted in an effort to raise primary parasitaemic bloodstream form populations bearing M-VATs (Schlappi and Jenni, 1977). Although this approach had worked successfully with T. brucei, with T. congolense there was no decrease in the prepatent period and the number of trypanosomes obtained was still poor. However, with some isolates this technique could work; for instance Crowe et al (1983) harvested 10^9 bloodstream forms expressing M-VATs nine days after feeding T. congolense infected flies on immunosuppressed mice.

A third method used the fact that trypanosomes present in local skin reactions in rabbits six to seven days post-infection appeared to express M-VATs. Those parasites could be used for serotyping

without further sub-passage (Luckins and Gray, 1979a). Using four stocks, the antigenic relationships of T. congolense originating from Kenya, Tanzania and Nigeria were examined by IFAT and the neutralization of infectivity test. Only two of the four stocks examined produced local skin reactions. However, impression smears from such reactions provided enough material to study two trypanosome poulations using antisera to the four stocks. From this work at least three serodemes of T. congolense were identified. The major disadvantage of this approach is the failure of some stocks to produce local skin reactions in rabbits.

Masake et al (1987) carried out a survey of T. congolense serodemes in Kilifi, Kenya. Twenty cattle were introduced into an area where they were in contact with G. austeni for six months. T. congolense was isolated from the cattle, passaged and cloned in mice to provide trypanosomes for further analyses. Trypanosome stocks and their cloned derivatives were inoculated into goats on which teneral tsetse flies were fed in order to provide metacyclics for serodeme characterization. Antiserum against metacyclics of a cloned trypanosome population was tested by IFAT using fresh unfixed tsetse salivary probes and a neutralization of infectivity assay. When an antiserum stained and neutralized all the metacyclics of the clone, that antiserum was tested against the parental stock. If complete neutralization occurred it was concluded that this stock contained only one serodeme and if there was incomplete neutralization the breakthrough population was cloned and analysed as for the parental stocks. The serodemes which were subsequently identified were confirmed by cross protection experiments in mice and goats. Four

serodemes from nine stocks were identified using this method and each of the nine stocks contained a mixture of at least two serodemes. In addition, the serodemes in the first isolates were also present in subsequent isolations six months later indicating that these serodemes were re-circulating during the study period and that immunity induced in the tracer cattle was not effective.

Apart from the work by Masake *et al* (1987) there is little information on the distribution and number of *T. congolense* serodemes from geographically defined areas. The present study was undertaken to examine the serodemes present in 17 stocks isolated from an area in Eastern Zambia near the South Luangwa game park. The use of *in vitro* culture systems enabled for the first time large numbers of antigenically stable metacyclic trypanosomes to be used in serological assays to examine serodemes of *T. congolense*. Four different assays were used to characterize the Zambian isolates: a cross protection assay; an ELISA using glutaraldehyde fixed, intact trypanosomes; indirect immunofluorescence was carried out using monoclonal antibodies to M-VATs of one isolate to determine the presence of specific M-VATs in the other isolates; and finally a triple labelling IFAT detecting the IgG response of the host to metacyclic trypanosomes was carried out using infection sera.

7.2 MATERIALS AND METHODS

7.2.1 Animals

Female outbred albino mice (TO) were used as hosts for the trypanosomes, to provide infective bloodmeals for tsetse flies and for cryopreservation of the parasites. Mice were also used in the cross protection assays involving infections with *in vitro*-derived

metacyclic forms. The mice were maintained by the method described in 3.6.1

Female NZW rabbits were used to maintain tsetse flies and to produce antiserum to the trypanosome stocks. The rabbits were maintained by the method described in 3.6.1.

7.2.2 Tsetse flies

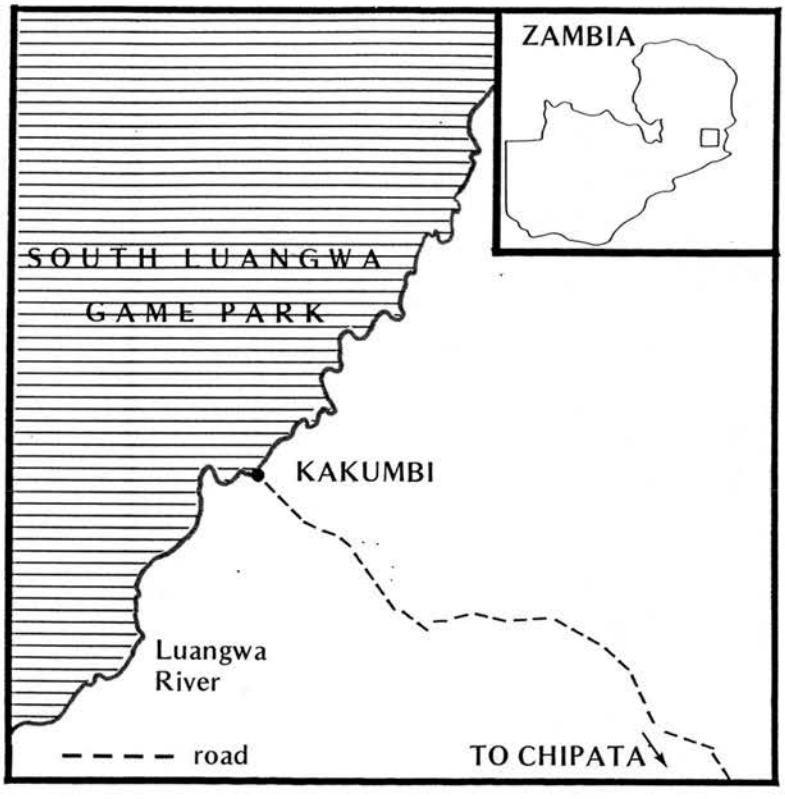
G. m. morsitans pupae were supplied by Dr. A.M. Jordan and were raised as described in 3.6.2. Flies were infected with the uncloned trypanosome stocks and used to transmit infections to rabbits.

7.2.3 Trypanosomes

The stocks of T. congolense used and their country of origin are shown in Table 7.1. Nine stocks were cultured in vitro six from Zambia (TREU 1881, TREU 1885, TREU 1894, TREU 1896, TREU 2034 and TREU 2037) and one each from Nigeria (TREU 1457), Tanzania (TREU 1676) and The Gambia (TREU 1627). These were used to supply metacyclic trypanosomes as reference antigens in the serological assays and to raise antisera in infected rabbits. The other 11 stocks shown in Table 7.1 were cyclically transmitted through G. m. morsitans and used to infect rabbits to produce antisera to those trypanosomes.

7.2.4 The study area

Stocks of T. congolense were isolated from domestic dogs used for hunting in a 320 ha survey area around Kakumbi in Chipata District, Zambia (Figure 7.1). The survey area lies close to the



scale: 1:150,000

FIGURE 7.1

The study area: Kakumbi, Chipata District, Zambia.

TABLE 7.1

Stocks of *Trypanosoma congolense* and their country of origin used in the experiments described in Chapter 7.

Trypanosome stocks used as antigens	Origin	Trypanosome stocks used to raise anti-sera from rabbits	Origin
* TREU 1881	Zambia	* TREU 1881	Zambia
* TREU 1885	"	* TREU 1885	"
* TREU 1896	"	* TREU 1896	"
* TREU 2034	"	* TREU 2034	"
* TREU 1894	"	* TREU 1894	"
* TREU 2037	"	* TREU 2037	"
* TREU 1457	Nigeria	TREU 1842	"
* TREU 1627	The Gambia	TREU 1851	"
* TREU 1676	Tanzania	TREU 1852	"
		* TREU 1457	Nigeria
		* TREU 1627	The Gambia
		* TREU 1676	Tanzania
		TREU 1844	Zambia
		TREU 1849	"
		TREU 1841	"
		TREU 1847	"
		TRPZ 118	"
		TRPZ 121	"
		TRPZ 106	"
		TRPZ 120	"

* denotes cloned stock

South Luangwa Game Park.

The vegetation of the Kakumbi area is dominated by large tracts of mopane woodland interspersed with more or less extensive mixed woodlands, thickets or clumps of trees. Open areas of grassland liable to flooding in the wet season, occur. In some areas, particularly nearer the road, but also deep in the bush there is scattered human settlement. There are no cattle or sheep but goats, dogs, chickens and ducks are present in some villages. Game animals are abundant even near the settlements.

Three species of tsetse occur in the area; Glossina morsitans morsitans and G. pallidipes are widespread, at high density in the undisturbed woodlands and at a lower density within and nearby the farms and villages. G. brevipalpis seems to be restricted to denser patches of thicket or riverine forest.

7.2.5 Cross protection assays

For each stock tested, eight groups of six mice were used. Six groups were infected intraperitoneally with 10^5 metacyclics of the stock being examined, one group acted as a positive control and was infected with 10^5 metacyclics of a known heterologous stock, TREU 1457, and the last group acted as a negative control and was injected with PSG on day 0. Between ten and 14 days post-infection the mice were drug treated. In the assays involving four stocks, TREU 2034, TREU 2037, TREU 1894 and TREU 1896 the mice in all eight groups were treated with 10 mg/kg Berenil (Hoechst Pharmaceuticals, West Germany). Assays involving the other two stocks, TREU 1885 and TREU 1881 used 0.5 mg/kg Samorin (May and Baker, U.K.) to treat the

mice because these stocks were resistant to Berenil at normal therapeutic doses. Fourteen days after chemotherapy the tail blood of all the mice were examined for infection to confirm that they were negative. Then, each of the six stocks was used to challenge a group of mice with 10^4 metacyclics intraperitoneally per mouse. The two control groups were challenged with the primary stock used to infect the mice. In addition to the eight experimental groups, two non-drug treated mice were infected intraperitoneally with 10^4 metacyclics of each stock used in the challenge. Presence or absence of parasitaemia was assessed by tail blood examination for up to 40 days post challenge.

7.2.6 The use of monoclonal antibodies to examine the relationships between stocks

Nine monoclonal antibodies which recognised M-VATs of T. congolense TREU 1885 and whose production and characterization were described in Chapter Four were used in a fluorescence assay (4.2.10) to examine the metacyclic populations of T. congolense, TREU 1881, TREU 2034, TREU 1894 and TREU 1896. The assay was carried out three times on acetone-fixed metacyclics. Each assay used metacyclics which had been prepared on separate occasions.

7.2.7 Examination of six Zambian stocks of T. congolense by ELISA

The relationships between TREU 1881, TREU 1885, TREU 1896, TREU 2034 and TREU 2037 were examined using the ELISA described in Chapter Five (5.2.4). Three, 21 day post-infection antisera for each stock were tested in duplicate at a dilution of 1:3200.

7.2.8 The use of a fluorescence assay to determine the relationships between stocks of T. congolense

The triple labelling method (6.2.10) was used in a series of three assays to determine the relationships between 17 stocks of T. congolense. Firstly, to ensure that the test would detect antigenic differences between the stocks cultured in vitro, 21 day post-infection antisera to TREU 1881, TREU 1885, TREU 2034, TREU 2037, TREU 1894, TREU 1896 were assayed against metacyclic trypanosomes of all of those stocks except TREU 1896. Then, serum against the uncloned parent stocks of TREU 1881, TREU 1885 and TREU 1896 (TREU 1842, TREU 1851 and TREU 1852 respectively) were assayed against the metacyclics of TREU 1881, TREU 1885, TREU 1896, TREU 1894, TREU 2034 and TREU 2037 to determine whether they recognized metacyclics other than those of their cloned derivative. Finally, antisera to eleven uncloned stocks isolated in the same area as the cultured stocks were assayed against the in vitro-derived metacyclics.

7.3 RESULTS

7.3.1 Cross protection assays in mice using in vitro-derived metacyclics of Trypanosoma congolense

The results of the cross protection experiments (Table 7.2) show that mice which were infected with metacyclic trypanosomes of one stock, drug treated with either Berenil or Samorin were immune to homologous challenge but succumbed to challenge by all the other stocks. A known heterologous stock TREU 1457 was used as a control and all the mice infected with this stock then drug treated were susceptible to challenge with all the stocks of Zambian origin.

TABLE 7.2

Cross protection assays in mice infected with T. congolense in vitro-derived metacyclics using six stocks, drug treated with Berenil or Samorin and then challenged with the stocks indicated.

Stocks used to infect mice	Number of mice showing protection when challenged with metacyclics of the stock indicated					
	TREU 1881	TREU 1885	TREU 2034	TREU 2037	TREU 1894	TREU 1896
TREU 1881	**6/6***	0/6	0/6	0/6	0/6	N.D.
TREU 1885	0/6	**6/6**	0/6	0/6	0/6	N.D.
TREU 1896	0/6	0/6	0/6	0/6	0/6	**6/6**
TREU 2034	0/4	0/6	**5/5**	0/6	N.D.	0/6
TREU 1894	0/6	0/6	0/6	0/6	**6/6**	0/6
TREU 2037	0/6	0/6	0/6	**6/6**	0/6	N.D.

N.D. Not determined
* Number of mice protected/number of mice challenged
Bold type signifies homologous challenge

Mice which were drug treated at the same time as the experimental mice but were uninfected also showed positive infections. The results indicated that all the cloned stocks of Zambian origin were not related antigenically and therefore the reference collection consisted of six different serodemes of T. congolense.

7.3.2 The use of monoclonal antibodies recognizing M-VATs of TREU 1885 in IFAT to examine metacyclics of other serodemes

None of the nine monoclonal antibodies which reacted with up to 84% of the M-VAT repertoire of TREU 1885 reacted with any of the metacyclic populations of four other serodemes TREU 1881, TREU 1894, TREU 1896 or TREU 2034 (Table 7.3).

TABLE 7.3

Results of immunofluorescence assays using monoclonal antibodies to metacyclics of T. congolense TREU 1885 and metacyclic trypanosomes, TREU 1885, TREU 1881, TREU 1894, TREU 1896 and TREU 2034.

Monoclonal antibodies	ANTIGEN				
	TREU 1885	TREU 1881	TREU 1894	TREU 1896	TREU 2034
TREUM 1.1	7.4%+	-	-	-	-
TREUM 1.2	8.5%+	-	-	-	-
TREUM 1.3	9.0%+	-	-	-	-
TREUM 1.4	17.0%+	-	-	-	-
TREUM 1.5	10.0%+	-	-	-	-
TREUM 3.1	7.6%+	-	-	-	-
TREUM 3.2	7.2%+	-	-	-	-
TREUM 3.3	6.6%+	-	-	-	-
TREUM 3.4	8.9%+	-	-	-	-

+ positive fluorescence
- negative

7.3.3 Determination of the relationships between stocks of Trypanosoma congolense using ELISA

The results of an ELISA which used in vitro-derived metacyclic forms and 21 day post-infection rabbit antiserum to those stocks are shown in Table 7.4. Absorbance values for homologous reactions were between 0.207 and 0.299. However, most heterologous absorbance values were not considerably different and in some reactions, for example those with anti-TREU 1881 and anti-TREU 1885 sera, the heterologous absorbance values were higher than homologous reactions.

TABLE 7.4

Results of an ELISA to examine the relationships between five stocks of T. congolense.

	ELISA values E_{450}^{nm} ±SD			
	ANTIGEN			
Antiserum (1:3200)	TREU 1881	TREU 1885	TREU 2034	TREU 2037
TREU 1881	***0.213 ± 0.087**	0.258 ± 0.186	0.308 ± 0.170	0.316 ± 0.136
TREU 1885	0.310 ± 0.177	**0.207 ± 0.102**	0.234 ± 0.204	0.299 ± 0.083
TREU 2034	0.193 ± 0.132	0.220 ± 0.057	**0.299 ± 0.097**	0.288 ± 0.098
TREU 2037	0.146 ± 0.045	0.300 ± 0.179	0.216 ± 0.058	**0.282 ± 0.135**
TREU 1896	0.276 ± 0.146	0.157 ± 0.087	0.228 ± 0.056	0.186 ± 0.068

* ELISA values based on replicates of three, 21 day post-infection sera

7.3.4 Determination of the relationships between stocks of Trypanosoma congolense using an immunofluorescence assay

The results of the triple label fluorescence assay are shown in Table 7.5. All the antisera showed positive fluorescence of greater than 90% of the population in the reactions between homologous metacyclic trypanosomes. No fluorescence was observed in heterologous reactions indicating that the serodemes were distinct and this was in agreement with both the cross protection assay and the fluorescence assay using monoclonal antibodies.

To examine the relationships between some of the uncloned parent stocks and the six serodemes, a fluorescence assay was carried out using antisera to three uncloned stocks, TREU 1842, TREU 1851 and TREU 1852. The results of this assay are shown in Table 7.6. Antisera to the parent stocks only reacted with the metacyclics of their cloned derivatives. This indicated that the parent

TABLE 7.5

The relationship between the six cloned stocks of T. congolense as defined by IFAT.

Antisera to: (1:160)	Rabbit number	ANTIGEN					
		TREU 1881	TREU 1885	TREU 2034	TREU 2037	TREU 1894	TREU 1457
TREU 1881	1645	+	-	-	-	-	-
	1661	+	-	-	-	-	-
	1662	+	-	-	-	-	-
TREU 1885	1667	-	+	-	-	-	-
	1687	-	+	-	-	-	-
	1682	-	+	-	-	-	-
TREU 2034	1614	-	-	+	-	-	-
	1610	-	-	+	-	-	-
	1680	-	-	+	-	-	-
TREU 2037	1660	-	-	-	+	-	-
	1657	-	-	-	+	-	-
	1656	-	-	-	+	-	-
TREU 1896	1490	-	-	-	-	-	-
	1481	-	-	-	-	-	-
TREU 1894	1837	-	-	-	-	+	-
	1805	-	-	-	-	+	-
	1836	-	-	-	-	+	-
TREU 1457	1500	-	-	-	-	-	+

TABLE 7.6

Results of a fluorescence assay using rabbit antisera to the uncloned parent stocks of TREU 1881, TREU 1885 and TREU 1896.

Antisera to: (1:160)	Rabbit number	Cloned derivative	ANTIGEN TREU 1881	TREU 1885	TREU 1894	TREU 2034	TREU 2037
TREU 1842	1798	TREU 1887	+	-	-	-	-
	1796		+	-	-	-	-
	1797		+	-	-	-	-
TREU 1851	1861	TREU 1885	-	+	-	-	-
	1855		-	+	-	-	-
	1853		-	+	-	-	-
TREU 1852	1880	TREU 1896	-	-	-	-	-
	1852		-	-	-	-	-
	1864		-	-	-	-	-

isolates contained only one of the five cloned stocks which were examined.

The assay was extended to examine eight other uncloned stocks isolated from the same area (Table 7.7). As with the other assays, the positive reactions were between homologous antisera and antigens indicating that the eight uncloned stocks were not related to the six serodemes used as the reference stocks.

7.4 DISCUSSION

Three serological assays using *in vitro*-derived metacyclic forms were used to determine the relationships between *T. congolense* isolates from Eastern Zambia. A cross protection assay in mice used living trypanosomes and the other two tests, ELISA and IFAT used glutaraldehyde and formalin-fixed metacyclics respectively.

TABLE 7.7

The relationship between uncloned Zambian isolates and the cloned reference stocks and defined by IFAT using in vitro-derived metacyclics as antigens.

Antisera to: (1:160)	Rabbit number	ANTIGEN TREU 1881	TREU 1885	TREU 2037	TREU 2034	TREU 1894
TREU 1844	1695	-	-	-	-	-
	1692	-	-	-	-	-
	1693	-	-	-	-	-
TREU 1849	1597	-	-	-	-	-
	1598	-	-	-	-	-
	1603	-	-	-	-	-
TREU 1841	1712	-	-	-	-	-
	1715	-	-	-	-	-
	1711	-	-	-	-	-
TREU 1847	1713	-	-	-	-	-
	1717	-	-	-	-	-
	1710	-	-	-	-	-
TRPZ 118	1701	-	-	-	-	-
	1703	-	-	-	-	-
	1707	-	-	-	-	-
TRPZ 121	1702	-	-	-	-	-
	1704	-	-	-	-	-
	1708	-	-	-	-	-
TRPZ 106	1763	-	-	-	-	-
	1795	-	-	-	-	-
	1799	-	-	-	-	-
TRPZ 120	1784	-	-	-	-	-
	1761	-	-	-	-	-
	1783	-	-	-	-	-
TREU 1881	1661	+	-	-	-	-
TREU 1885	1682	-	+	-	-	-
TREU 2034	1614	-	-	-	+	-
TREU 2037	1656	-	-	+	-	-
TREU 1894	1800	-	-	-	-	+

+ positive fluorescence
- negative

The cross protection assay was based on observations that the mammalian host can be successfully immunized against homologous but not heterologous trypanosomes (Luckins and Gray, 1983; Akol and Murray, 1983; Nantulya *et al*, 1984). When mice were infected with cultured metacyclics of the cloned Zambian stocks, then drug treated and challenged with other *in vitro* derived metacyclic forms, they demonstrated immunity only to the infecting stock (Table 7.3) indicating that the cultured metacyclic reference collection consisted of six different serodemes. The advantages of this assay are that it uses living trypanosomes and is therefore free from possible fixation artefacts. It also uses small numbers of trypanosomes; 10^5 metacyclics per mouse for the primary infection and 10^4 per mouse for the challenge. There are, however, disadvantages in that the test uses large numbers of laboratory animals, it is time consuming and cumbersome since the trypanosomes have to be prepared on the day of infection/challenge followed by a 40 day screening period. In addition, the test is limited by the number of stocks producing metacyclic forms *in vitro*. Another method of using viable trypanosomes to examine variant antigens is the neutralization of infectivity test (Wilson and Cunningham, 1972; Goedbloed *et al* 1973; Dar *et al* 1973; and Masake *et al* 1987). The advantage of this assay over the cross protection test is that a reference collection can be assayed against a wide range of infection sera. However, like the cross protection test, trypanosomes have to be prepared on the day of the assay and the ability of the trypanosomes to infect laboratory mice has to be ensured. Wilson *et al* (1973) examined only seven *T. congolense* isolates out of a total of 145 because of poor

infectivity of the trypanosomes for laboratory mice.

The use of fixed trypanosomes produced mixed results in the serological assays. Although the ELISA previously detected differences in M-VAT repertoires of different trypanosome stocks (Chapter 5), when the test was applied to metacyclics of the Zambian stocks and antisera to those stocks, no differences in absorbance values were observed (Table 7.5). This could be due to poor antigen preparation and/or one or several non-specific factors present in anti-trypanosome rabbit serum.

Monoclonal antibodies raised to M-VATs of one stock, TREU 1885, did not react with any of the M-VATs from four stocks isolated in the same area (Table 7.3). Although over 80% of the TREU 1885 M-VAT population was recognised by the monoclonal antibodies, this approach was of limited value compared to using polyspecific antisera in analysing the relationships between M-VAT repertoires of seveal serodemes. Monoclonal antibodies would have to be prepared against most of the M-VATs of many serodemes to be of greater practical value. However, the specificity of monoclonal antibodies has many advantages over polyclonal serum. In particular, they have enabled examination of the composition of populations of T. congolense (Crowe et al, 1983; Prain and Ross, 1988) and T. b. rhodesiense (Esser et al, 1981; Turner et al, 1988).

Immunofluorescence was the most successful of the serological assays using fixed and intact metacyclic trypanosomes. This assay detected differences in trypanosome M-VAT repertoires which were indicated using viable trypanosomes in cross-protection assays. The metacyclics from in vitro culture were used as reference stocks. Two

assays were carried out. The first assay examined the relationships between the uncloned parent stocks from which the cloned reference stocks were derived. The results indicated that each of the parent stocks consisted of only one of the six serodemes in the reference collection. This could be due to the repeated passage in mice of the original isolates and thus only the trypanosomes which were infective to mice would be selected. Alternatively, the number of serodemes circulating in the area could be so large and diverse that the probability of recognising more than one of the reference collection would be small. The results from the second assay using antisera from eight uncloned stocks would support the suggestion that a large number of serodemes were present in this survey area. Antisera from animals infected with the uncloned stocks produced negative results in the immunofluorescence assay indicating that those stocks were not related antigenically to the metacyclics of the reference collection. Therefore, from the results of the serological assays, it appears that at least seven serodemes were present amongst the 17 stocks isolated from this area although there are probably many more.

Early work on isolates from four different areas in East Africa suggested that there were many different antigenic variants circulating both between geographically separate areas in Uganda, Tanzania and Kenya and within one area in Kenya (Wilson et al, 1973). However, antigenic relationships were observed between stocks isolated from the four areas surveyed and those variants persisted over the 15 month study period. The authors recognized that the results of tests using primary parasitaemia populations and the

neutralization of infectivity test had to be accepted with considerable caution and that techniques involving metacyclic forms should be used.

The characterization of serodemes circulating on a ranch at Kilifi, Kenya was achieved using metacyclic trypanosomes in immunofluorescence and neutralization of infectivity tests. Four distinct serodemes were identified from eight stocks and seven clones. Furthermore, each isolate contained a mixture of at least two serodemes. At Kilifi there was minimal stock movement and game migration around the ranch. The level of tsetse transmitted trypanosomiasis was considered to be low, mainly consisting of T. congolense and arising from an isolated focus of G. austeni (Masake et al, 1987; Paling et al, 1987).

In comparison, Kakumbi is located close to the boundary of the South Luangwa Game Park where tsetse and game are abundant. Therefore, extensive game movement and large numbers of possible reservoir hosts of T. congolense would be expected. This could explain the, possibly, large numbers of serodemes present in Kakumbi. Although three species of tsetse were present, G. morsitans morsitans, G. pallidipes and G. brevipalpis, it has been suggested that the dogs may have become infected by scarification during feeding on wild game (Godfrey, personal communication). If the dogs were infected by non-tsetse transmitted means, it raises the question of what importance these trypanosomes were for tsetse infected domesticated livestock. Perhaps only a proportion of the serodemes present in the dogs would be present in the domestic livestock. However, the number of T. congolense serodemes which

could potentially infect tsetse flies and livestock might be large.

The idea of a local vaccine based on a 'cocktail' of metacyclic antigens has been proposed by many workers since the identification of limited M-VAT heterogeneity (Vickerman and Barry, 1982; Murray et al, 1979; Masake et al, 1987). This 'cocktail vaccine' theory has always depended on the identification and enumeration of trypanosome serodemes circulating in nature. The results of the serodeme characterization of T. congolense isolates from Kakumbi obtained in this study and earlier studies on T. brucei, T. vivax and T. congolense, suggest that the number of antigenic variants circulating even in relatively small areas is indeed large (Goedbloed et al, 1973; Wilson et al, 1973; Dar et al, 1973). Moreover, tsetse flies may inoculate a mixture of trypanosome serodemes and species on a single occasion thus complicating the situation still further (Dar et al, 1973). Areas such as Kilifi where there is an isolated tsetse fly focus of a single species, where there is little game or stock movement within the area and where T. congolense is the main trypanosome species affecting domestic livestock are probably rare. Added to the problems of identifying such an area is the possibility of genetic recombination between trypanosomes of different serodemes producing new variant antigens (Jenni et al,1986). Therefore, strict monitoring of serodemes circulating would have to occur over a period of time before any evaluation of control measures could be taken. Vaccination based on a 'cocktail vaccine' of local serodemes is therefore not a feasible proposition.

There is evidence however, that livestock maintained under routine drug therapy in certain areas may acquire a degree of

resistance to trypanosomiasis (Bevan, 1928, 1936; Wilson et al, 1975, 1976; Bourn and Scott, 1978). This could be due to a limited and stable number of serodemes circulating in such an area and it is conceivable that sterile immunity could develop if an animal under constant challenge is antigenically primed before the trypanosomes are killed with the drug (Whitelaw, Bell, Holmes, Moloo, Hirumi, Urquhart and Murray, 1986).

In some studies with isometamidium chloride (Samorin, May and Baker), the drug was used prophylactically and therefore immunity played no part in enhancing protection (Robson, 1962; Fairclough, 1963; Trail et al, 1985). There is evidence that some stocks of T. vivax have a relatively low sensitivity to the prophylactic activity of Samorin but a higher sensitivity to therapeutic activity (Peregrine, Moloo and Whitelaw, 1987). If this observation was also true for some T. congolense stocks then a more efficient method of chemotherapy with Samorin might include using the drug therapeutically, allowing immunity to develop rather than relying on chemoprophylaxis alone.

It has been suggested that the duration of chemoprophylaxis may be directly related to the level of challenge (Whiteside, 1962) and groups of cattle have developed a limited immunity to reinfection as judged by the reduced necessity for further treatments (Wilson et al, 1975, 1976). Therefore, if areas were identified where the circulating trypanosome serodemes were identified and found to be stable, it might be possible for animals to acquire immunity by allowing them to become infected before using strictly controlled

drug regimes. This approach would not be feasible however, where the number of circulating serodemes was high and constantly changing.

CHAPTER EIGHT

GENERAL DISCUSSION

Epidemiological studies have potential value in several aspects of African animal trypanosomiasis including the acquisition of information on the ecology and natural history of the disease and in the determination of the origins of disease outbreaks. Information on the epidemiology of trypanosomiasis is important not only in the planning and monitoring of disease control programmes but also in assessing the economic effects of the disease and analysis of the benefits of control programmes (Habtemariam, Ruppanner, Riemann and Theis, 1983; Thrusfield, 1985).

The epidemiology of the tsetse-transmitted animal trypanosomiases is the result of a complex series of interactions between trypanosomes, tsetse flies and vertebrate hosts. In domesticated livestock, three species of trypanosomes, T. congolense, T. vivax and T. brucei are capable of causing disease and although infections with a single species of trypanosome have been reported, there is a likelihood of multiple infections occurring due to superinfection with the same or different species or simultaneous transmission with more than one species (MacLennan, 1970; Willett, 1970). The trypanosomes are capable of being transmitted by as many as 30 different species or subspecies of tsetse fly each of which is adapted to different climatic conditions and epidemiological niches. Trypanosomes are primarily parasites of wild game animals such as

non-domesticated ungulates, carnivores, and to a lesser extent, primates. The game animals also provide the principle food source for the tsetse flies. Domesticated animals, principally cattle, are infected with trypanosomes when they compete with the game animals for land and thus come into close contact with tsetse and interrupt the sylvatic cycle.

Although T. congolense is one of the most important aetiologic agents of animal trypanosomiasis, its epidemiology is poorly understood. Variations within T. congolense in such characteristics as infectivity to tsetse flies, morphology, pathogenicity and mammalian host range have all been identified and could be of epidemiological importance (Godfrey, 1961; Mulligan, 1970; Hoare, 1972; Stephen, 1986). However, these characteristics are difficult to define and may be subject to external influences such as species of vector transmitting the parasite, mammalian host species and their nutritional state, and exposure to trypanocidal drugs. To be of value in epidemiological studies a characteristic should be able to be defined and be stable. Such characteristics as genetic diversity (Majiwa et al, 1985; Majiwa et al, 1986) isoenzyme profiles (Young and Godfrey, 1983; Gashumba, 1986; Gashumba, Baker and Godfrey, 1988) and antigenic repertoires (Wilson et al, 1973; Masake et al, 1987) have all been used in attempts to distinguish between different isolates of T. congolense.

Isoenzyme (Gashumba et al, 1988) and DNA hybridization (Majiwa et al, 1986) analyses of T. congolense have identified four distinct groups; East African savanna, East/West African savanna, riverine/forest and Kenya coast which broadly correspond to the geographical

and ecological isolation of those stocks examined. In contrast, a number of studies attempting to characterize T. congolense isolates serologically have shown evidence of a greater diversity within this species (Wilson et al, 1973; Masake et al, 1987).

In addition to the multiple variable antigen types expressed during a single infection, there is also an unknown number of serodemes each capable of expressing a different repertoire of variable antigens. Although a large number of VATs are capable of being expressed by the bloodstream form trypanosomes early in infection, predominant VATs (P-VATs) appear in a predictable sequence (Gray, 1965b). Therefore, these P-VATs could be isolated and characterized in serological assays and used to identify and enumerate trypanosome serodemes. However, although this approach is applicable to the T. brucei subgroup trypanosomes which can be easily cloned and maintained in laboratory animals (Van Meirvenne et al, 1977), it is not easily applied to T. congolense because of this parasite's poor infectivity and low virulence for laboratory animals. This was shown particularly well in attempts by Wilson et al (1973) to serologically characterize isolates of T. congolense where only seven of the 145 isolates could be examined using trypanosome populations raised in mice.

Metacyclic trypanosomes are introduced into the skin of the mammalian host during feeding by an infected tsetse fly and are therefore the first stage in the trypanosome life cycle to be encountered by the host. The M-VATs expressed by these trypanosomes are limited in number compared to those in the bloodstream of an infected

host and the M-VAT repertoire is characteristic for any given serodeme (Crowe et al, 1983).

In cattle, antibodies to M-VATs have been shown to appear by day 16 post-infection (Akol and Murray, 1985). Thus, by comparing M-VAT repertoires, using the specific immune response of the host to metacyclics, serological typing of T. congolense serodemes could be achieved. Unfortunately, tsetse flies infected with T. congolense produce few metacyclics and attempts to raise bloodstream form trypanosomes expressing M-VATs in laboratory animals are often thwarted by antigenic variation making it difficult to obtain the large numbers of trypanosomes required for serological assays. Other attempts to use trypanosomes expressing M-VATs in local skin reactions in rabbits (Luckins and Gray, 1979a) and goats (Dwinger et al, 1987) were limited by the failure of some stocks to produce local skin reactions.

In this study, serodemes of T. congolense were characterized using in vitro-derived metacyclic trypanosomes as reference antigens. Recent developments in the in vitro-cultivation of T. congolense have enabled different developmental stages of this parasite to be maintained in the laboratory free from host and vector influences (Gray et al, 1981; Gray et al, 1984; Gray et al, 1985; Ross et al, 1985). The metacyclic trypanosomes which are produced in large numbers on a regular basis from cultures of epimastigote forms are morphologically, biologically and immunologically identical to the metacyclic forms found in the tsetse fly (Luckins et al, 1981; Crowe et al, 1983; Prain and Ross, 1988). In addition, metacyclic trypanosome populations produced over long periods in culture remain antigeni-

cally stable (Gray and Luckins, 1982; Luckins et al, 1986; Prain and Ross, 1988). Therefore, in vitro-derived metacyclics would appear to be ideal for use as antigens. However, at the outset of this project, there were few stocks in culture and there was no collection of trypanosomes isolated from a geographically defined area. Although a number of isolates of T. congolense which had previously been shown to be antigenically distinct were available in culture, they were from widely separated geographical regions. Hence, it was necessary to establish in culture the stocks isolated from an area in Eastern Zambia. This also gave an opportunity to determine the in vitro characteristics, including metacyclic production, of trypanosomes from Central Africa.

Each stock showed wide variation in the numbers of metacyclics produced in vitro, a possible indication of infraspecific variation shown by T. congolense. It is also possible that different serodemes might also exhibit different characteristics in relation to their infectivity to tsetse flies, infectivity and pathogenicity in mammalian hosts and in their sensitivity to trypanocidal drugs.

There is evidence to suggest that the VAT repertoires of some serodemes of T. b. rhodesiense and T. b. gambiense are not distinct and that certain M-VATs are expressed in more than one serodeme (Vervoort et al, 1983; Barry, 1986; Turner et al, 1988). Monoclonal antibodies prepared against one isolate of T. congolense did not recognize any M-VATs in other cultured stocks suggesting that each of these serodemes was distinct. Further analysis of the isolates by cross-protection assays and IFAT confirmed both their distinctive antigenic repertoires and those of another 11 isolates from the same

area. From these assays at least seven serodemes were identified from 17 stocks.

There is very little known about the distribution of serodemes for any trypanosome species. T. b. gambiense appears to have one major serodeme prevalent throughout West and Central Africa (Gray, 1972; Jones, Cunningham, Taylor and Gray, 1981) although minor serodemes have also been detected (Pays, Dekerck, Van Assel, Babiker, Le Ray, Van Meirvenne and Steinert, 1983c). A restricted number of serodemes may also exist in T. b. rhodesiense. In a focus on the shores of Lake Victoria in East Africa, one serodeme has been responsible for recurring epidemics of sleeping sickness over a 20 year period although, as with T. b. gambiense, there is at least one other minor serodeme present in this area (Barry et al, 1983; Barry, 1986). Geographical and ecological barriers have probably isolated T. b. gambiense from T. b. rhodesiense so that they have evolved leaving discrete serodemes associated with distinct epidemiological niches, although the overlap observed in some VAT repertoires for both subspecies probably makes serodemes difficult to define.

T. congolense however, has less restrictions on distribution than T. b. rhodesiense and T. b. gambiense making the characterization of serodemes on a geographical basis much more difficult than for the subspecies within the Trypanozoon subgenus. The large and diverse game reservoir and various tsetse fly species ensures that serodeme diversity of this parasite could be immense.

The serodemes characterized in this study were isolated from domestic dogs used for hunting. There was no evidence from bloodmeal analyses that the three species of tsetse fly present in this area,

G. m. morsitans, G, brevipalpis and G. pallidipes were feeding on dogs and it was assumed that they were infected by scarification during feeding on infected game animals. Since the flies were not feeding on dogs, it seems unlikely that these animals were an important reservoir for the tsetse transmission of the T. congolense serodemes identified. However, the results of this study do indicate the diversity of serodemes present in game animals in this study area.

An important, but rarely addressed feature of serodeme distribution is the influence of the vector and its feeding habits in transmitting infections to different animal species. Different species of Glossina have specific feeding habits and available hosts are used discriminately as a food source of tsetse flies (Weitz, 1970). For example, G. m. morsitans feeds preferentially on suids (30-50% of all feeds) and particularly warthogs. The principle bovids (25-40% of all feeds) fed on by G. m. morsitans are kudu, buffalo, bushbuck and eland. G. brevipalpis feeds preferentially on bushpig (40%) and hippopotamus (36%) whilst G. pallidipes obtains its bloodmeals mainly from the bushbuck (80-90% of all feeds). Therefore, although the overall number of serodemes appears to be large, that number may be restricted within particular game-tsetse life cycles. Furthermore, since domesticated livestock, particularly cattle, are not fed on preferentially by tsetse flies, the distribution of serodemes in cattle may be further restricted. Cattle have been shown to survive on the shores of Lake Victoria in the presence of G. fuscipes but not G. pallidipes indicating that G. fuscipes is less important in transmitting T. congolense to cattle than G. pallidipes (Jordan,

1986). This could be related to the infectivity of the serodemes present in this area for G. pallidipes in comparison to their infectivity for G. fuscipes. Therefore, when identifying T. congolense serodemes circulating in a defined geographical area it would be advisable to examine the serodemes present in the tsetse flies and to identify the mammalian host species on which they feed. This would be particularly important when assessing the serodemes infecting cattle or other domesticated livestock.

It is important to monitor the presence and distribution of serodemes over a period of time. T. congolense serodemes circulating in an isolated focus such as at Kilifi, Kenya will remain stable over long periods, thus producing an endemic situation. However, areas such as this ranch are probably uncommon and game movement and certain animal husbandry practices such as transhumance and trekking cattle to market will enable different serodemes to be introduced into new areas thereby creating epidemics of infection. Monitoring of game and cattle movement in conjunction with serodeme characterization may predict areas where epidemics are likely to occur.

Another factor which might influence the expression of particular M-VAT repertoires is the frequency of transmission of a limited number of serodemes such as might occur in areas with a tsetse population feeding almost exclusively on cattle. Under these conditions there might be a need for modification of the M-VAT repertoire. In the field, repeated challenge with the same VATs would lead to immunity and the curtailment of transmission of that serodeme. Therefore, expression of new VATs may enable establishment of the parasite in the mammalian host and ensure the survival of that

particular serodeme. In experimental infections with one serodeme of T. b. rhodesiense, changes in the M-VAT repertoire including the expression of novel M-VATs have been observed (Barry et al, 1983). It seems unlikely however, that a few changes in the M-VAT repertoire would be sufficient to ensure continued transmission of the serodeme and changes in the early bloodstream form VAT repertoire would also be necessary.

Existing control measures, namely tsetse control by insecticide spraying and treatment of animals with trypanocidal drugs, are effective only when the control programmes are carefully planned and managed. Excellent veterinary supervision is required with trained personnel and adequate funding. Many livestock projects are marginal in economic terms and the occurrence of trypanosomiasis can mean that they easily become unprofitable. The cost of arranging regular surveillance by blood sampling, adherence to a strictly timed and accurately dosed drug regimen, often with the need to finance periodic activities to reduce the number of tsetse present ensure that some projects become loss makers (Ford, 1971).

Areas of tsetse infestation can be easily determined by trapping the flies, identifying the species and then plotting their distribution. This can be achieved on a local scale or on a national basis. Having identified the areas where tsetse need to be controlled, residual or non-residual insecticides may be applied. However, while tsetse flies may be eradicated in certain areas, few regions have circumscribed boundaries and unless cleared areas are defended, re-invasion of the flies invariably occurs thus limiting the effects of this control measure.

Only five drugs are currently available for the treatment of tsetse-transmitted animal trypanosomiasis and no new drugs have been marketed since 1960 (Jordan, 1986). Improper use of the available drugs, for example underdosing and infrequent use, has led to the widespread development of drug resistance. In practice, in many African countries, only two drugs, diminazene aceturate and isometamidium chloride are still being used. Ideally, chemotherapy should only be undertaken when livestock are exposed to occasional risk of trypanosomiasis such as during the seasonal occupation of tsetse infested areas or on the margins of fly infested areas. Where chemotherapy is practised while livestock are continually at risk, drug resistance is likely to occur. Once drug resistance is acquired by trypanosomes they remain resistant to the drug even when they return to the sylvatic cycle and are no longer exposed to that drug (MacLennan, 1980).

The alternative approach to chemotherapy is chemoprophylaxis, but to be successful it needs even stricter supervision of drug administration and conditions of animal husbandry than does chemotherapy. To be an effective prophylactic, a drug must be slowly eliminated from a treated animal. It is important to administer a second dose of the drug before the level in the tissues drops to such a level that resistance might develop. Such a regime requires the continued presence of trained staff, reliable transport and access to the animals involved. Chemoprophylaxis is therefore inappropriate for use with nomadic or semi-nomadic livestock in pastoral management systems (Jordan, 1986).

The Mkwaja ranch in Tanzania is often held to be an example of what can be achieved in areas of high trypanosome challenge by successful veterinary supervision of control measures (Trail et al, 1985; Jordan, 1986). On this ranch, cattle are treated prophylactically with isometamidium chloride and therapeutically with diminazene aceturate. Strict monitoring of cattle for evidence of infection and careful adherence to drug treatment has resulted in a productivity index which is 80% that of Boran cattle maintained in non-tsetse areas: despite this carefully applied regime the level of productivity achieved is economically non-viable. In addition, recent reports suggest that the drugs are having to be administered more frequently indicating that resistance might be developing. Hence this entire approach to controlling trypanosomiasis on the ranch might be in jeopardy.

An alternative method of control of trypanosomiasis is drug-stimulated immunity which could possibly be linked to tsetse trapping techniques to reduce fly density. This phenomenon is poorly understood but fieldwork by Wilson et al (1975, 1976) indicated that the success of this method is directly related to the intensity of trypanosome challenge. In areas of high challenge, the development of immunity during drug therapy is unlikely and this could be related to the number and stability of the serodemes circulating. However, in areas of medium and low challenge and when serodemes in the area are few in number and stable, this approach might be successful. An analysis of serodemes circulating in an area such as the Mkwaja ranch could allow control measures which use drugs less frequently thus reducing the possibility of inducing drug resistance.

Unfortunately, national governments and international aid agencies have short term goals, implementing existing control measures to limit the effects of the disease. A longer term approach examining the basic epidemiology of the disease including tsetse infestation, trypanosome species infecting the flies, drug sensitivities of those trypanosomes and the serodemes present in cattle and tsetse flies feeding on those cattle could lead to a more rational approach to the control of the disease. However, as the infrastructures at national and regional levels are often incapable through lack of resources of implementing current control measures, it is unlikely that the funding and coordination necessary to implement a detailed examination of all the factors mentioned above could ever be achieved. Nevertheless, if the problem of drug resistance continues to increase and no new drugs become available, alternative strategies of control may need to be examined. In such circumstances, acquired resistance to a limited number of serodemes possibly linked with the elimination of tsetse flies important in the transmission of those serodemes could be envisaged.

It is clear that the epidemiology of the tsetse-transmitted animal trypanosomiases is multifactorial and that antigenic diversity within T. congolense is only one of many parameters which could be examined. This study has described the development and application of serological techniques using metacyclic trypanosomes from in vitro culture systems to examine the distribution and diversity of T. congolense stocks isolated from a small area in Eastern Zambia in 1981. The initial isolation of the stocks to in vitro culture, the production of monoclonal antibodies and the development of the ELISA

and IFAT which distinguished M-VAT repertoires using fixed trypanosomes meant that this particular study was limited to examining stocks isolated from dogs. Nevertheless, having established those techniques it would now be possible to examine serodemes present in tsetse flies, and game animal species in this area and to study the changes in serodemes over time. Furthermore, this technique could be applied to any chosen area once a reference collection of metacyclic trypanosomes was established *in vitro*.

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APPENDIX I

FIGURE 1 KAKUMBI, ZAMBIA, 1981

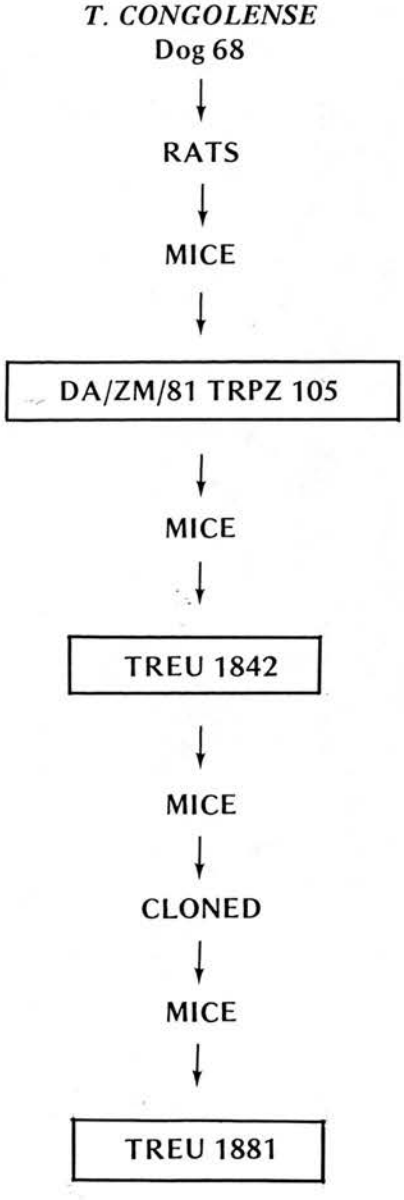


FIGURE 2 KAKUMBI, ZAMBIA, 1981

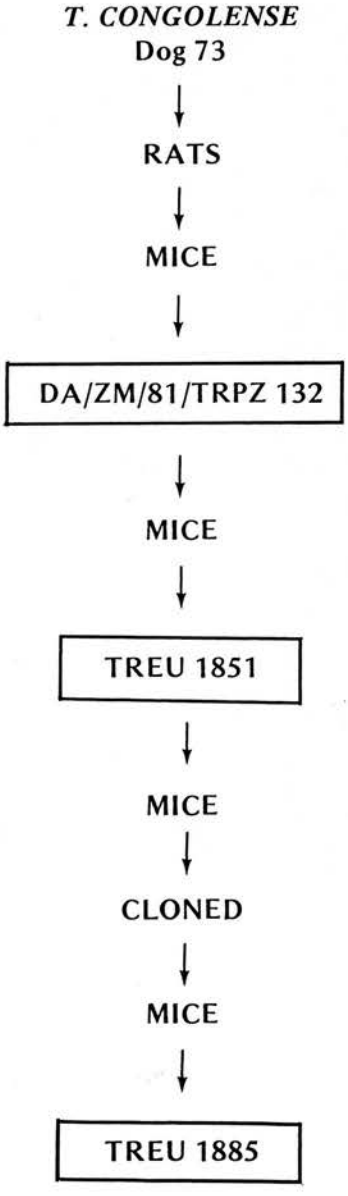
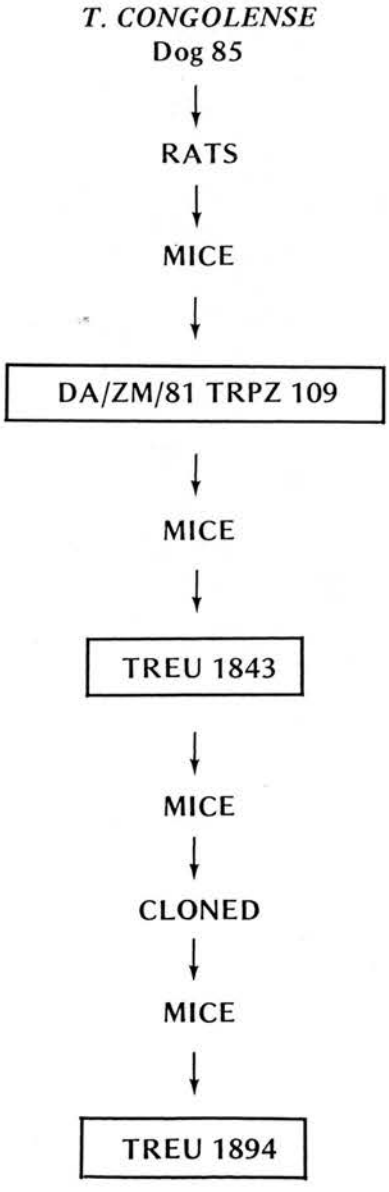


FIGURE 3 KAKUMBI, ZAMBIA, 1981



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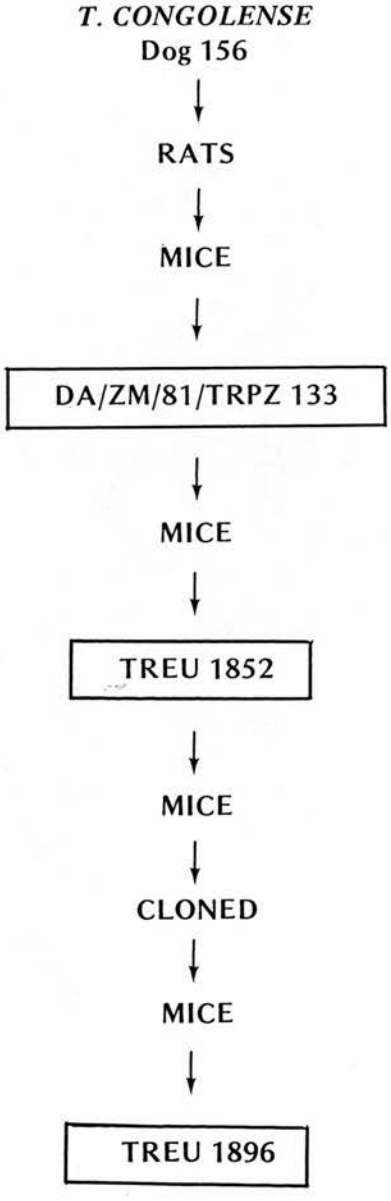

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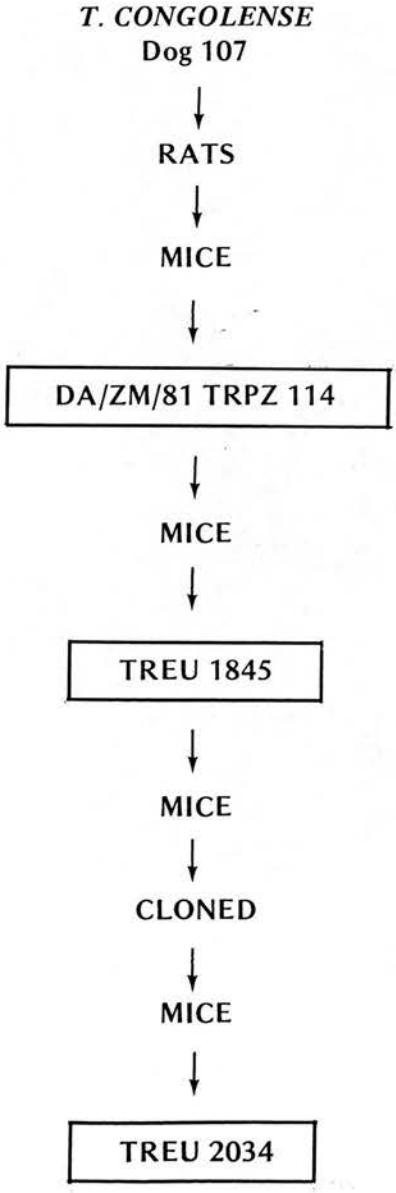

FIGURE 6 KAKUMBI, ZAMBIA, 1981